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14. ABSTRACT
Over the last 12 months of this award, we have focused on developing an in-depth understanding of the immune system in the setting of the tumor microenvironment. We have made progress in developing methods to better analyze the relationships between primary tumor and metastatic growths with their surrounding microenvironments, and implications for immune response and dendritic cell function *in vitro* using 3D microculture techniques. We have further investigated and tested alternative methods for tumor eradication using combinatorial drugs in attempts to restore/enhance the immune response. Additionally, we continue preparing breast cancer cell lines to aid quantifying progress in upcoming *in vivo* studies using mouse models. We anticipate that the progress made in these last 12 months will lead to combining observations into *in vitro* and *in vivo* models to better test our combinatorial immunotherapeutic strategies, restore dendritic cell function in cancer, and identifying novel tumor-associated stroma targets.

15. SUBJECT TERMS
Breast Cancer, immunotherapy, tumor microenvironment, dendritic cells, metastasis, cancer stroma.

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INTRODUCTION:

The immune system and cancer are both complex biological systems that interact and affect each other. While there have been recent successes in cancer immunotherapy including PROVENGE, a dendritic cell based vaccine for prostate cancer, and antibodies blocking immune checkpoints (CTLA-4 and PD-1) for melanoma, these have produced clinical benefits only in a subset of patients. The intimate relationships between cancer cells, immune cells, and tumor associated stromal cells must be explored and investigated in order to truly have an effective immunotherapy for breast cancer. It has more recently become clear that not only does the immune system respond to cancer cells, but the process goes both ways, with cancer also able to suppress the host immune system. Tumor-infiltrating lymphocytes (TILs) have been shown to be functionally impaired in many cancers (*1*). In tumors where TILs were found to be functional, the prognosis was consistently favorable (*2, 3*). The collective data suggest that T cell infiltration—when functionally active—leads to a favorable outcome in breast cancer. These data concerning the tumor environment complement our own findings that changes in immune cells within tumor-draining lymph nodes (TDLNs) strongly correlate with clinical outcome in breast cancer (*4*). Despite the complexity, certain elements can be teased out and focused upon for maximal impact. Our previous studies have led to key insights into the mechanisms behind the immune dysfunction that breast cancer causes. Comprehending how the different phases—activation, expansion and effector functions—of a normally functioning immune response are disrupted in the presence of breast cancer will allow us to develop strategies to counteract the problems and restore immune function to optimal levels in patients. This focus on unraveling the dynamics between breast cancer and host immune system in a comprehensive and systematic manner is the underlying principle of my goal to develop rational combination immunotherapy for breast cancer, one that is truly effective long term at eliminating metastases and thereby preventing relapse in breast cancer patients. In order to build on the observations my lab has made with regards to cancer-dendritic cell function and immune cell-cancer relationships, immune responses within the tumor microenvironment must be analyzed in depth to identify unique markers, molecular and cytokine signals, and associated cell populations which may be aiding in immunosuppression. To this end, I have built a strong research team for this project, which includes assistant research professor Dr. Brile Chung, PhD postdoctoral fellows Dr. Dobrin Draganov and Dr. Neta Zuckerman, and research associates Sara Moeller and Gayathri Srinivasan. We worked closely with clinical collaborators at the City of Hope under an IRB approved protocol. In this annual report, I will discuss the foundation being laid toward the goals outlined in our statement of work, and the approaches that we will test to remedy the global immune dysfunction in breast cancer.

BODY:

We have a strong research team for this project, which includes assistant research professor Dr. Brile Chung, PhD postdoctoral fellows Dr. Dobrin Draganov and Dr. Neta Zuckerman, and research associates Sara Moeller and Gayathri Srinivasan. We worked closely with the CoH IRB office on a human subject's protocol which has been approved. We have close collaborations with breast cancer surgeons, pathologists, and the tissue bank at CoH in order to procure breast cancer tissue, lymph node, and blood samples for our analyses. Animal work will begin soon under an animal protocol (IUCAC #13042) which was approved at City of Hope and recently approved by the DoD in August 2014. In addition, we are in the process of submitting a second IACUC protocol to be approved by City of Hope (IACUC #14040). Our team has been working hard on establishing cell lines, protocols, and preliminary experiments to build a solid foundation and direction for progressing towards the tasks in our statement of work. Listed below are the main aims which we proposed, corresponding tasks from our statement of work, and our progress to-date.

Aim 1. Enhance efficacy of dendritic cell (DC)-based vaccination by promoting DC maturation and clustering in vivo.

Task 1. Identifying mechanisms by which breast cancer disrupts DC clustering and maturation : months 1-48.

Task 2. Testing novel strategies to enhance DC clustering *in vivo*: months 1-48.

Aim 2. Enhance T cell function *in vivo* by restoring immune signaling.

Task 3. Investigating mechanisms by which chronic IL6 affects immune function: months 1-36.

Task 4. Testing the effectiveness of IL6-blockade plus IL27 treatment in reversing chronic IL6-induced T cell dysfunction: months 12-48.

Aim 3. Select optimal integrated immunotherapy combinations in animals for clinical development.

Task 5. Select Optimal Integrated Immunotherapy Combinations in Animal Models for Clinical Development: months 12-60.

Results:

<u>Aim 1. Enhance efficacy of dendritic cell (DC)-based vaccination by promoting DC maturation and clustering *in vivo.*</u>

Task 1. Identifying mechanisms by which breast cancer disrupts DC clustering and maturation: months 1-48.

a. Test effects of known breast cancer secreted molecules on DC clustering and maturation (month 1-36).

b. Identify novel breast cancer secreted molecules on DC clustering and maturation using proteomics and gene expression analysis after laser capture micro-dissection (month 1-48).

In order to gain knowledge on the molecules and factors which influence DC clustering and maturation, especially in the context of cancer, we first would need to establish a better understanding of the dynamics between immune cells and the tumor microenvironment. To this end, we have worked to establish an *in vitro/ex vivo* model which helps mimic cell-cell interactions in a 3D microenvironment. It has been established in recent years that 3D cell culture environments provide a more physiological representation of cell interactions. 3D

cultures also produce markers and behavior that might not be seen on traditional monolayer culture experiments, and applies also specifically to the breast cancer setting (*5*). The tumor microenvironment is comprised of heterogeneous populations of cells including cancer, immune, and cancer-associated stromal cells (CAS) to name a few. Clinical data and experimental models have shown that the extent and nature of immune infiltrations into tumors is an important independent prognostic factor (*6*). Recent findings suggest that CAS is another important regulator of tumor growth and progression which may also modulate the recruitment, activation status, and retention of immune cells in the tumor microenvironment (*7*). Therefore, targeting the CAS plus cancer cells is essential for the success of cancer immunotherapy.

Progression of tumor growth and initiation of metastasis is critically dependent on the reciprocal interactions between cancer cells and tumor associated stroma. CAS have been known to promote inhibitory effect on T cells by producing various factors and cytokines such as TGF beta, VEGF, HGF, IL-6, and IL-17 (*8*). These factors also may play a very important role in understanding the characteristics of DC's in the tumor microenvironment. Here, we have developed 3D cell aggregate culture system that recapitulates the human tumor microenvironment by incorporating essential populations of CAS to investigate the dynamic relationship between the tumor microenvironment and the immune system. Our novel approach focuses on separate expansion and later re-aggregation of the subpopulations of primary human breast cancer stroma and immune cells that are critical for supporting or inhibiting cancer growth. A major advantage of our tumor aggregate system is that cancer, stromal, and immune cells can be easily manipulated *in vitro* (etc. numbers, mixture of ratio, or engineered) (Figure 1). Furthermore, we will be performing genomic profiling of cancer cells and CAS to identify differential gene expression patterns in these reconstructed co-cultured cancer cell/stromal cell 3D organoids. (Figure 2)

Optimized culture conditions for growth of patient-specific breast cancer cells and CAS for generation of tumor microenvironment 3D-organoids.

Conventional methods to develop an *ex vivo* system that resembles the microenvironment within human tumors are suboptimal. Highly passaged cancer cell lines grown in two-dimensional (2D) monolayer systems do not recapitulate the 3D *in vivo* microenvironment within tumors, and do not reflect the molecular and cellular mechanisms involved in proliferation, differentiation, and metastasis of cancer cells. Knowledge of cell-cell and cell-extracellular matrix interactions within 3D spatial environment is critical for understanding the complex cross-talk mechanisms that enhance cancer cell growth and immune evasion *in vivo* (*9-11*). Equally important is the interaction between cancer cells and their CAS (*12, 13*). A critical gap in our understanding of this interaction during breast cancer progression is lack of a 3D breast cancer microenvironment composed of not only viable cancer cells derived from primary or metastatic patient samples, but also the stromal cells derived from that same patient. To address this need, we have made substantial progress in successfully growing breast cancer patient-specific cancer cells and CAS (from both primary and metastatic tumors), then using these cells to generate organoids. Our method involves growing primary breast cancer cells in a way that is less physically and chemically damaging to tissue and cells, and maintains their dependence on endogenous stromal cells from the same tumor from which the cancer cells are derived. We believe preserving this dependence is essential for maintaining growth of primary cancer cells *ex vivo* such that the cells retain their *in vivo* biology, and therefore is a major advance over current methods In addition, to further replicate the complex TME *ex vivo*, we have developed methods to form 3D multicellular organoids (*14*) consisting of matched cancer and stromal cells (and also immune cells). This enables further studies of the complex genomic and molecular changes that take place within the 3D tumor microenvironment. In the proposed studies, we will optimize both *ex vivo* growth of cancer and stromal cells, as well as formation of organoids. To develop our method, breast tumor samples were cut into fragments via mechanical dissociation and plated directly onto tissue culture-treated dishes. This tissue was cultured for 2 to 3 weeks, during which breast cancer cells (EpCAM/CD326+, non-adherent) and CD326-/CD44+/CD140B+ (adherent) CAS both expanded in the culture (Figure 3). However, stromal cells have the propensity to overtake cancer cells when co-cultured over time. To maximize the growth of CD326+ cancer cells without expanding CAS simultaneously, we plated sorted CD326+ cancer cells onto autologous CAS that

have been treated with either mitomyocin or sub-lethal irradiation (Figure 4A-I). To further promote the expansion of cancer cells, specific culture conditions that preferentially support the separate *ex vivo* growth of human epithelium and stroma were established (Figure 4A-II & 4A-III). In the epithelium-specific culture, sorted CD326+ cancer cells will be cultured in calcium- free DMEM supplemented with 1% FBS, cholera toxin (10 ng/ml), bovine insulin (3 μg/ml), hydrocortisone (0.5 μg/ml), EGF and V-valine. Shown in Figure 4B are the results of FACS analyses of CD326+ CD184+/- expressing cancer cells expanding in epithelium-specific culture condition without the presence of stroma. Furthermore, we also observed expansion of trans-differentiating cells like mesenchymal stem cells (MSCs) differentiating into adipocytes (oil red positive) adjacent to fibroblasts, demonstrating that heterogeneity was maintained in our cultured stromal population (Figure 5). We will also generate various patient-specific CAS lines by using our tumor tissue fragment culture technique, including CAS populations derived from metastases to organs such as brain, bone, and lymph nodes.

Identification of differential gene expression and alternatively spliced mRNA variants involved in different cancer associated stroma populations.

The main objective of this study is to identify genes/splice variants and pathways that are involved in cancer-stroma crosstalk within the 3D tumor microenvironment. Many gene transcripts expressed in breast cancer cells are alternatively spliced (*15*) and exhibit splicing patterns that may be markedly distinct from CAS. Such differences may affect the outgrowth of each cell population in organoids, and have implications for communication among cancer cells and CAS. Differences in expression of alternatively spliced RNAs cannot be detected by standard cDNA microarray analysis because cDNA microarrays lack the internal probe sets required to identify alternatively spliced internal exons. Alternatively spliced transcripts expressed in cancer and stroma can be detected by next generation sequencing. The Illumina RNA-seq platform can detect differently expressed alternatively spliced exons, which represent a rich, previously untapped source of molecular signatures that can be used to distinguish subtle changes in the transcriptome in the tumor microenvironment. Here, we are particularly interested in genes and splice variants of genes that exhibit changes in expression during stromal-cancer interaction, and the influence of this heterogeneity on growth of organoids. We will also determine if heterogeneity that is present in the original tumor tissue is still present in the organoids. This analysis is expected to identify many exon-specific gene markers (or exon signature sequences) that have not been detected in previous global analyses of gene expression. These exon sequences may prove useful for detecting subtle changes in gene expression patterns depending on cell type in 3D organoids, and have implications for improving generation of organoids. To perform genomic analyses, we generated individual human breast stromal aggregates (normal, primary, or metastatic) and purified the RNA separately (Figure 6B) for human exon specific microarrays and next generation sequencing. Pathway Analysis will be used to identify pathways/genes that are enriched in stroma and cancer cells. Integrating these two genomic technologies will allow us to generate a complete transcriptional landscape, which will greatly benefit our long term goal of identifying molecular signatures for characterizing cell types and targets for therapeutic purposes. The results of exon microarray analysis and next generation sequencing will be used to i) characterize stromal and cancer cells, ii) create a database of unique genes/exon signatures that are concordantly over (or under) expressed in all cancer, CAS and normal stromal/epithelial cells, and iii) identify genes and pathways that are involved in stroma-tumor coupling in primary and metastatic state, for which perturbation can result in therapeutic endpoint. These results will establish a better understanding of the dynamics between immune cells and the tumor microenvironment in order to determine the molecules and factors which influence DC clustering and maturation.

To identify and validate potential cancer-related genes which are up or down regulated, we have also performed RT-PCR gene expression profiling on normal stroma and CAS. Normal stroma were obtained from patients undergoing prophylactic measures for preventative purposes. We have performed quantitative RT-PCR on cancer-associated stroma (BC68) and normal stroma (BC78) aggregates, using a cancer gene panel purchased from Bio-Rad. qPCR analysis revealed several cancer associated genes that were up regulated in CAS as compared to normal stroma. Notable genes that were upregulated includes, KDR, MET, IGF1, and CDK2N (Figure 6A**).** Further experiments will be performed to understand how knocking down and inducing

down regulation of these genes will affect the cross-talk between CAS and cancer cells in the 3D organoid setting and how this influences DC clustering and maturation.

Breast cancer secreted molecules on DC clustering and maturation using proteomics and gene expression analysis

In order to better evaluate the molecules or factors that may be affecting DC clustering and maturation, we must be able to re-create a suitable method for recapitulating the tumor microenvironment in a controlled setting. The 3D culturing techniques presented and used identify a more physiological method (versus monoculture *in vitro*) for identifying the interactions between tumor microenvironment and immune cells. The method of using 3D aggregate cultures will allow us to create an isolated environment of PBMC derived DCs along with tumor associated stroma or healthy stroma cells, and with or without primary tumor involvement if desired. The cells then can be analyzed via histological analysis, as demonstrated previously, from paraffin embedded 3D aggregate cultures to identify DC populations. Laser capture microdissection can then be utilized on these 3D aggregate sections and run for analysis of genetic expression changes of the DCs exposed to tumor associated stroma versus healthy stroma. Futhermore, results can be compared to genetic expression microarray profiles available in public domain. Specifically, utilizing a deconvolution methodology, which was developed by our lab (*16*), we are able to perform analysis of cell-type specific gene expression using existing large pools of publically available microarray datasets and identify if the trends found in our 3D aggregate analysis for DC populations is identified in other tumors/cancers (*16*). We also have begun banking tumor associated stromal RNA, healthy stromal RNA, as well as tumor RNA for future analysis on microarray or RNAseq to identify unique markers which may be influencing DC clustering or maturation as well as other potential implications to surrounding immune cells in the tumor microenvironment.

Task 2. Testing novel strategies to enhance DC clustering *in vivo*: months 1-48.

a. Test agents known to modulate DC clustering and maturation in vitro (month 1-24).

b. Test molecules that block factors identified in task 1 (month 12-48).

The maturation status of dendritic cells (DCs) plays an important role in determining the nature of an immunologic response. Mature DCs are capable of eliciting an effective “immunogenic” response. An effective anti-tumor response is elicited when DCs present tumor antigens to T cells leading to activation and proliferation of specific T cells. Over the past decade, there has been considerable effort made to characterize DC populations in cancer. A pivotal location to examine such immune-tumor interactions is the tumor-draining lymph node (TDLN). It is the site where tumor antigens are typically first presented to the immune system and a critical initial decision between immune activation and tolerance is made. We have previously shown that DCs in healthy lymph nodes (HLNs) tend to aggregate in large clusters of mature cells, whereas DCs in TDLNs tend to remain either un-clustered or form smaller clusters with fewer mature cells. This clustering behavior promotes functionality of DCs by increasing the number of proximal T cells compared to un-clustered DCs. Intriguingly, the degree of DC clustering within TDLNs correlated strongly with better clinical outcome in breast cancer patients.

Plans for the next 12 months:

- Knock down experiments of KDR and MET will be performed to address cross-talk between CAS and cancer cells in the 3D organoid system

- Continue integrating multiple cell types involved in *in vitro* 3D tumor microenvironment to determine factors which affect tumor progression and metastasis.

- Attempt to identify potential markers on stromal cells or cancer cells which may be producing unique immunosuppressive properties.

- Perform analysis on tumor associated and healthy stroma in the tumor microenvironment by microarray or RNAseq to help identify unique genetic expression profiles or functional defects which may influence tumor progression/metastasis.

- Test possible agents which may modulate immunosuppressive conditions in 3D microenvironment and expand 3D culture aggregate system with use of 3D hydrogel matrix.

- Monitor effect of modulation of DC clustering/maturation by testing known agents previously investigated and mentioned above, by utilizing assays in 3D aggregate cultures and 3D hydrogel matrix systems.

Supporting Data/Figures:

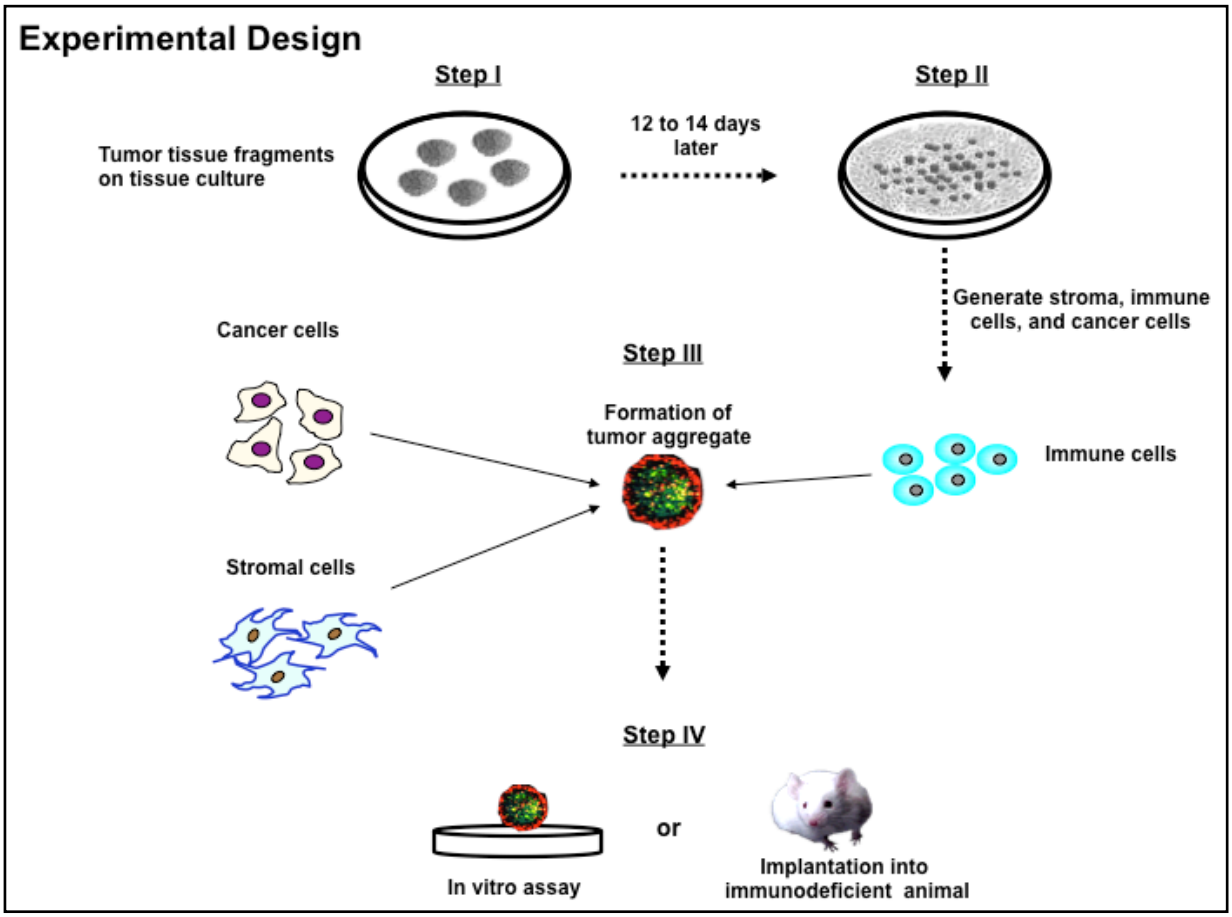


Figure 1- Experimental Design: Illustration of the steps involved in producing 3D aggregate populations from tumor associated cells and use *in vitro* or potentially *in vivo.*

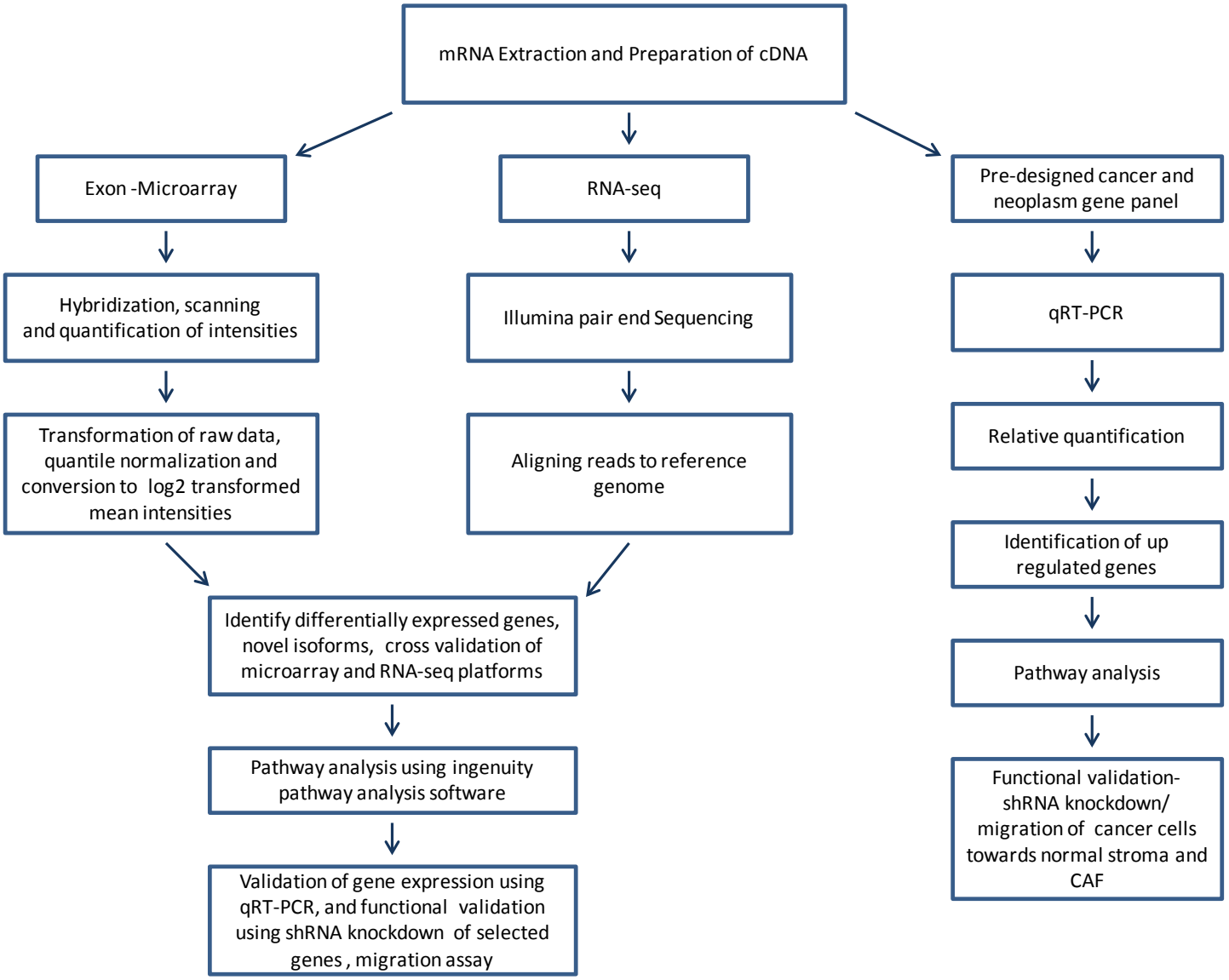
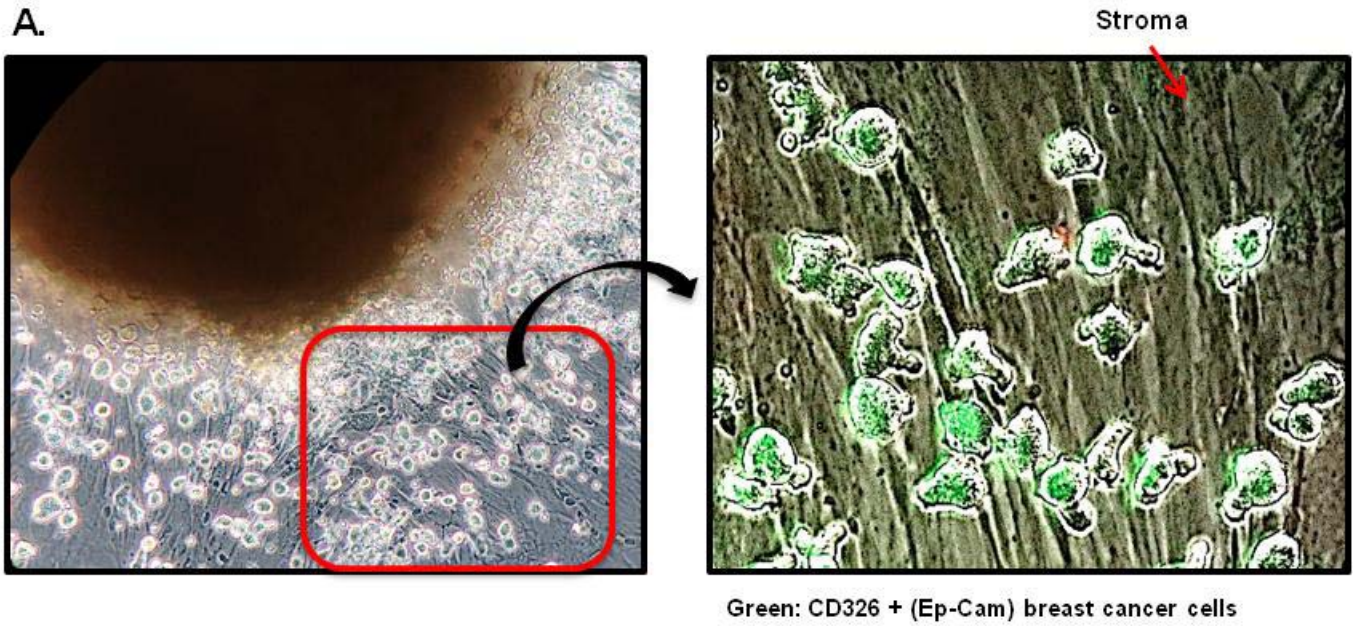


Figure 2: Experimental Design. Illustration of the steps involved in genomic profiling of cancer cells and CAS to identify differential gene expression patterns in the reconstructed co-cultured cancer cell and stromal cell 3D organoids.



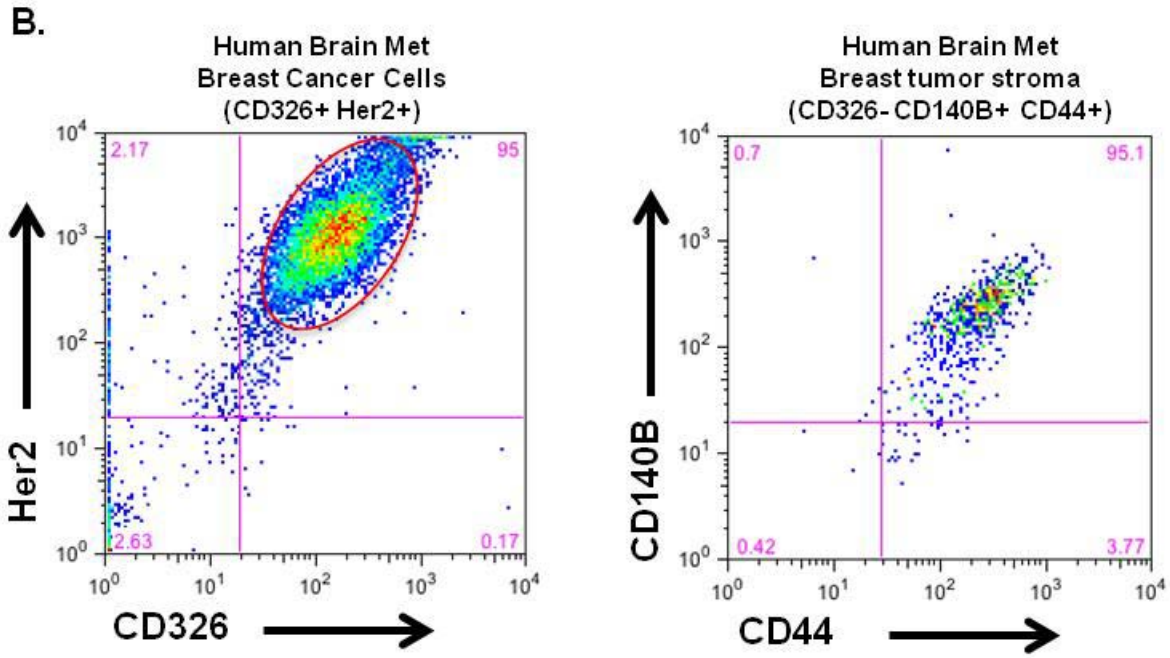


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Figure 3. Expansion of breast cancer patient-specific cancer and stromal cells. (A) Left, patient breast tumor fragment plated directly onto culture flasks. Right, co-expanded breast cancer cells (green) and CAS (arrow) after 3 weeks in culture. (B) Characterization of breast cancer cells and CAS via FACS.

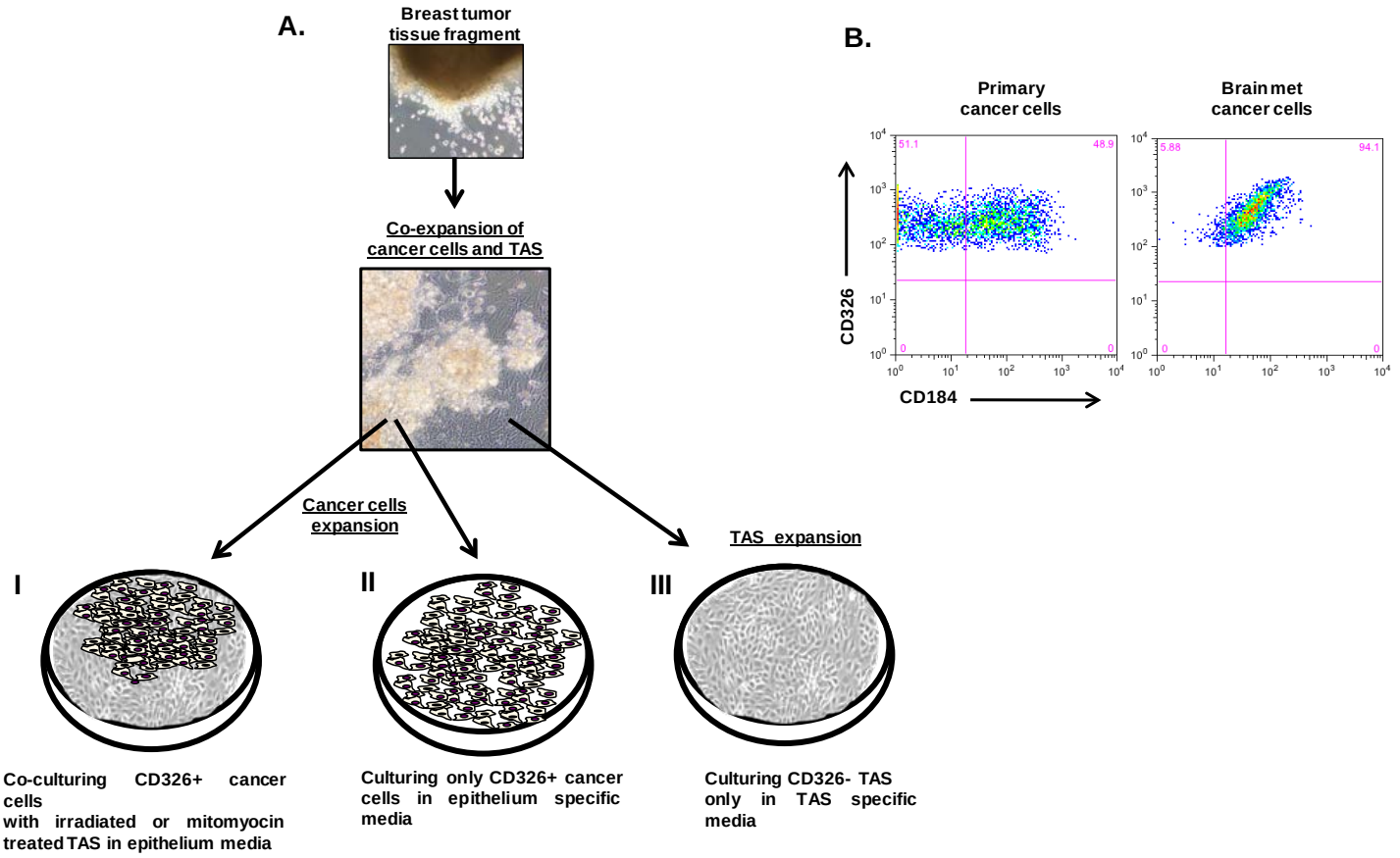


Figure 4. Effective optimization of culture conditions for growing cancer cells and CAS from breast cancer tissue. (A) Experimental design to promote the expansion of cancer cells. Shown here are specific culture conditions that preferentially support the separate *ex vivo* growth of human epithelium and stroma (**Figure 4-II & 4-III**). (B) The results of patient-specific primary and brain met breast cancer cells growing *in vitro* without the presence of CAS.

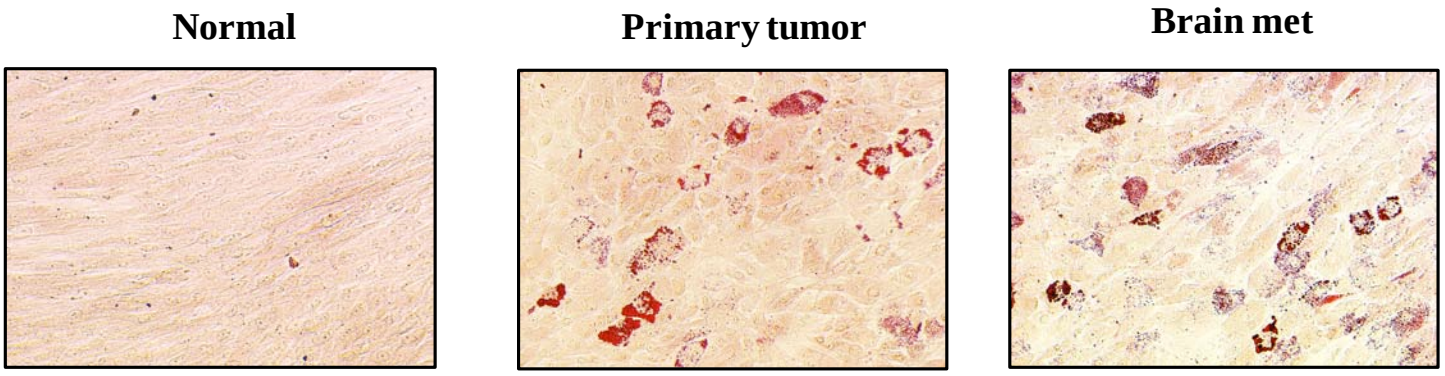
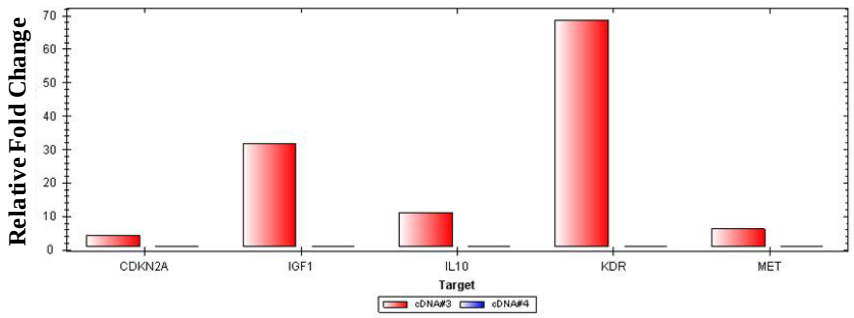


Figure 5. Presence of trans-differentiating stromal cells in expanded cells from primary and metastatic tissue. Oil red staining (which identifies adipocytes) of expanded stromal cells from normal, primary tumor, and metastatic tumor (brain).

A.



Target	Fold change in the gene expression of BC68 (cDNA3) relative to BC78 (cDNA4)
CDKN2A	4.46212
IGF1	31.81692
IL10	11.24132
KDR	68.72192
MET	6.31714

B.

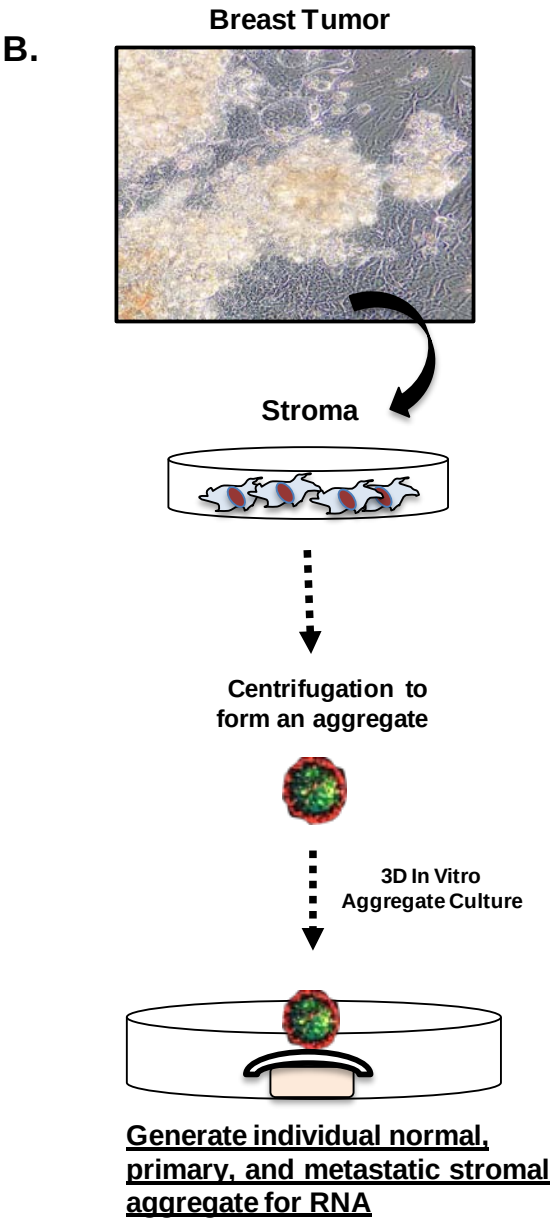


Figure 6. Gene expression profiling of normal and primary associated stroma from 3D cell structure. (A) Relative expression of cancer associated genes in CAS and normal stroma. The data denotes fold gene expression using the-ΔΔCT method. (B) Schematic for generation of human 3D breast tumor aggregate models (organoids) *in vitro*.

Aim 2. Enhance T cell function *in vivo* by restoring immune signaling.

Task 3. Investigating mechanisms by which chronic IL-6 affects immune function: months 1-36.

a. Gene expression analyses and flow cytometry to measure expression of positive and negative signaling regulators (month 1-36).

b. T cell polarization and functional assays (month 1-36).

Task 4. Testing the effectiveness of IL-6-blockade plus IL-27 treatment in reversing chronic IL-6-induced T cell dysfunction: months 12-48.

a. In vitro assays to test the effects of treatment with an anti-IL-6 antibody plus IL-27 during naive CD4 T cell polarization and CD8+ T cell activation (month 12-36).

b. Test various treatment strategies to reverse T cell dysfunction in BC patients (month 24-48).

Our focus over the past year has been on Aim 1 and Aim 3, as some of the developments from these Aims would enable us to pursue Aim 2 in a more efficient and comprehensive way. Aim 2 will be pursued in the coming year with the following plans.

Plans for the next 12 months:

- Measure IL-6 cytokine levels from sera and evaluate T cell responses to IL-6 and IL-27 in a new cohort of breast cancer patients.
- Assess the functional consequences, e.g, cell survival, proliferation, and T cell polarization, of defective IL-6 signaling.
- Potential agents which may enhance or restore cytokine balance (IL-6/IL-27 responsiveness) will be analyzed in 3D aggregate culture. Including investigation of stromal cell impact on cytokine signaling consequences on multiple cell types in the tumor microenvironment.
- Continued gene expression data to be collected in specific tumor conditions to identify the potential impact cytokine regulation has on multiple types of T cells and their ability to function as well as potential DC clustering/maturation impact.
- IL-6 blockade will be further assessed *in vitro* so that *in vivo* analysis can be carried out appropriately.

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Aim 3. Select optimal integrated immunotherapy combinations in animals for clinical development.

Task 5. Select Optimal Integrated Immunotherapy Combinations in Animal Models for Clinical Development: months 12-60.

While some combinations of FDA approved cytokine therapeutics for cancer (IL-2 and IFN-α2b) have shown modest incremental efficacy, they have been limited by substantial toxicities (*17*). Previous cytokines tested clinically as cancer therapeutics were selected based on putative effects to enhance immune function rather than specifically to correct immune signaling defects in cancer. In addition, DC vaccination effectiveness may be hampered by an immunosuppressive environment, which prevents maturation and clustering in lymph nodes. Each of the key phases of the immune response—induction, amplification, and effector immune cell generation—is defective in breast cancer patients, so it follows that integrated immunotherapy combinations that address all of the phases of the normal immune response will be much more effective than individual treatments that address only one mechanism.

Developing integrated immunotherapeutic regimens to target the immune signaling defects that occur in each phase of the immune response (Figure 7), in combination with optimal DC-vaccination strategies using immunologically validated antigens, will generate highly functional and prolonged anti-tumor immune responses in breast cancer patients that will prevent recurrence and metastasis.

We will focus on developing a thorough approach to best take our findings about the 3D tumor microenvironment and its unique setting with our knowledge of cytokine signaling functions in immune and cancer cells and then attempt to assess *in vivo* effectiveness of combining the regimens for induction of optimal anti-tumor immunity. Then we will determine the optimal time to administer these regimens during disease progression, with and without chemotherapy. As surgery removes the primary tumor burden, we will focus our studies on the post-surgical setting where we envision immunotherapy is most effective to eradicate micrometastases to prevent relapse.

Last year's report concentrated largely on tasks 5a-5c for optimization purposes. These last 12 months, we have focused primarily on task 5d.

Task 5a. Optimize post-surgical murine model of breast cancer metastasis (month 12-36).

Task 5b. DC vaccination optimization by restoration of DC clustering and maturation (months 24-48).

Task 5c. Optimization of the amplification and effector phases by correcting chronic IL6-mediated defective T cell responses . (month 24-48).

Task 5d. Testing other strategies and combinations. (month 48-60).

Doxorubicin is a known inducer of immunogenic cell death, and long-term protection is critically dependent on both direct cytotoxicity and immune-mediated mechanisms. Key characteristics of doxorubicin-induced immunogenic cell death include Calreticulin exposure and the release of high-mobility group protein B1 **(**HMGB1) and adenosine triphosphate **(**ATP) proteins. Recently, autophagy has been linked to the release of ATP from tumor cells undergoing immunogenic cell death. We have identified the FDA-approved drug Ivermectin (IVM) as a potent agent that can kill mouse and human breast cancer cells alone and is also highly synergistic in combination with Doxorubicin (Figure 8). Ivermectin induces rapid and largely necrotic cell death that, contrary to immunologically "silent" apoptosis, has the potential to be extremely immuno-stimulatory. ATP plays a key role in immunogenic cell death and regulates multiple immune processes, including the migration of phagocytes and the activation of T cells and antigen presenting cells (*18*). Based on the reported

ability of Ivermectin to modulate ATP-gated purinergic signaling (*19*), we hypothesized that the synergistic Doxorubicin-IVM combination could be a promising strategy to enhance immunogenic cell death and anti-tumor immunity. Our analysis indicates that the synergistic killing of breast cancer cells is dependent on autophagy. Contrary to previous reports, the direct anti-tumor toxicity of IVM is not mediated through the generation of reactive-oxygen species. Instead, drug IVM-induced autophagy, ATP release, Ca2+ signaling, and ROS seem to be protective, at least in the context of short-term high-dose exposures. We were unable to confirm any causal role for ROS in killing under extended lower-dose exposures either. Blocking P2X7 receptors and NADPH oxidase, however, potently inhibited cell death, suggesting a dual role of ROS and purinergic signaling and the existence of parallel death pathways downstream of exaggerated or sustained P2X7/NADPH activation. The therapeutic potential of this novel strategy is further supported by our preliminary data suggesting promising immuno-modulatory effects, including augmented Teff/Treg ratios, targeting of myeloid derived suppressive cells (MDSCs), and enhanced antigen-presenting cell (APC) responses.

Previous reports on the anti-leukemia effects of IVM alone or in combination with Doxorubicin were attributed to enhanced uptake of Doxorubicin, cell swelling and induction of reactive oxygen species. IVM also synergized with other ROS-inducing chemotherapeutic agents, both *in vitro* and *in vivo*. The ability of IVM to modulate purinergic signaling and its immunomodulatory potential, however, were not addressed in any of these earlier studies. In fact, generation of ROS is an event that is known to occur downstream of ATP signaling and is responsible for activation of the inflammasome pathway in antigen presenting cells. We were able to confirm the ability of IVM to potentiate inflammasome activation and secretion of IL-1β from LPS-primed murine dendritic cell line (DC2.4), but not from murine 4T1.2 breast cancer cells (Figure 9).

To investigate whether ATP signaling and the inflammasome pathway might be involved in the killing of 4T1.2 breast cancer cells by IVM, we first confirmed that exogenous ATP enhanced IVM killing. Importantly, IVM killing could be effectively blocked by the specific P2X7 inhibitor KN-62 (Figure 10). P2X7 receptors are frequently over expressed in various cancers, including breast cancer, and are known to promote tumor growth but are also capable of inducing tumor cell death. Paradoxically, depletion of extracellular ATP with apyrase or blockade of ATP release through the PANX-1 channels potentiated rather than suppressed IVM killing of 4T1.2 cells, consistent with a dual role of purinergic signaling (Figure 11). It seems that despite the ultimate synergistic killing, ATP release is an early defense mechanism protecting breast cancer cells from immediate IVM-mediated killing. It has been argued that autophagy is a similar protective response against cell damage caused by various chemotherapeutic agents. One of the hallmarks of immunogenic cell death is the release of ATP in response to treatment with chemotherapeutic agents such as doxorubicin. Recently, it has been shown that this ATP release is critically dependent on autophagy. We therefore investigated whether autophagy, and ATP release, might be involved in the synergy between Doxorubicin and Ivermectin. Using autophagy deficient variants of the human MDA-MB-231 breast cancer cells, we were able to show that synergy was indeed dependent on autophagy (Figure 12). Moreover, several known inducers of autophagy (Rapamycin, Bortezomib, Resveratrol, Wortmannin) were all able to synergize with IVM in killing of 4T1.2 cells. Rapamycin, however, inhibited immediate high-dose IVM killing (4h), consistent with an early protective role of autophagy and ATP release (Figure 13). Interestingly, IVM itself was able to induce autophagy (Figure 14), which might potentially be further enhanced by combination with the other synergistic drugs.

We were interested if Ivermectin might be able to modify the mode of cell death or have an impact on the hallmarks of immunogenic cell death (Figure 15). Unlike Doxorubicin, which induces delayed apoptotic cell death, higher dose IVM kills tumor cells quickly through largely necrotic or secondary necrotic mechanism (Figure 16). High dose IVM-induced cell death is therefore more likely to be immunostimulatory. Inhibitors of several known cell death pathways were able to inhibit killing, making it difficult to identify the primary mode of IVM-induced cell death (Figure 17). Although we were unable to see enhanced uptake of Doxorubicin or dramatic increase in cell size, the involvement of caspase-1 and the inflammasome point to a pyroptotic rather than apoptotic mechanism of IVM-induced cell death for apoptosis, which is associated with Chloride efflux and cell shrinkage rather than cell enlargement (Figure 18 and 19). Further experiments will clarify the primary

mode of cell death in the context of extended exposure to synergistic low doses of Doxorubicin and Ivermectin. We anticipate that addition of IVM will enhance the characteristics of Doxorubicin induced immunogenic cell death. Although IVM induced only a marginal up-regulation of surface calreticulin (CRT) exposure (Figure 20), the increase of surface mannose-6-phosphate (M6P) expression was significant (Figure 21). Autophagy induced up-regulation of M6P has recently been demonstrated to confer sensitivity of tumor cells to granzyme B and CTL-mediated lysis.

We previously demonstrated that IVM concentrations of 5μM and lower are generally non-toxic to various PBMC subset populations of cells. To establish the therapeutic window and the tumor specificity of IVM treatment, we compared the sensitivity of human and mouse cancer cell lines to normal human foreskin and mouse embryonal fibroblasts, respectively. IVM concentrations of 8uM specifically target only mouse cancer cells (Figure 22). The synergistic combination of doxorubicin and IVM is also specific for human breast cancer rather than normal fibroblasts (Figure 23). On the other hand, extended exposure to IVM concentrations above 10μM is toxic to all cell types, defining a very narrow therapeutic window for IVM as a single agent, as previously reported in leukemia. The therapeutic window for IVM-based therapies, however, can be significantly augmented by the ATP rich tumor microenvironment, secondary drugs inducing autophagy, or immune mediated effects.

The central role of ATP signaling in immune regulation suggested possible immunomodulatory and potentiating effects of Ivermectin. Concentrations of IVM that are directly toxic to tumor cells (4-16 uM) might be able to preferentially deplete immunosuppressive populations such as Tregs and MDSCs. Normalizing the dysfunctional T cell and myeloid compartments is a prerequisite for a successful cancer immunotherapy. Our preliminary data show that *ex-vivo* IVM enhances the Teff/Treg ratios in human PBMCs (Figure 24). Moreover, these potentiating effects are reversed by exogenous IL-2 but are more pronounced in the context of concomitant T cell stimulation or exposure to high exogenous ATP, suggesting that IVM could be a useful tool to enhance antigen specific responses (Figure 25). Future experiments utilizing the Ovalbumin OT-1/OT-2 system will help us to clarify that question further. The observed higher sensitivity to IVM of splenic MDSCs isolated from tumor bearing mice will be another direction for future investigation.

In summary, we have been able to demonstrate that Ivermectin is a novel and promising therapy for breast as well as other solid cancers, where it can be used as a single agent or in combination with autophagy-inducing therapeutics such as, doxorubicin, rapamycin, bortezomib, etc., IVM is particularly promising because its direct anti-tumor cytotoxic effects can be further expanded through the induction of immunogenic cell death and boosting anti-tumor immune responses. Our next step will be the evaluation of the therapeutic potential of IVM in the 4T1.2 murine metastatic breast cancer model *in vivo*. The *in vivo* data will be used to identify the most optimal synergistic combination of IVM for further clinical development. IVM has a strong potential to be synergistic with a number of FDA approved cancer drugs such as, those mentioned above, as well as targeted therapeutics such as tamoxifen, EGF/Her2 blocking antibodies and inhibitors, all of which are potent inducers of autophagy. These standard of care autophagy inducing anti-cancer drugs might be insufficient to kill tumor cells alone, but will likely enhance IVM-mediated killing, thus overcoming the development of drug resistance.

Plans for the next 12 months:

- Determine the primary mode of cell death in the context of extended exposure to synergistic low doses of doxorubicin and Ivermectin.

- Evaluate the therapeutic potential of IVM in the 4T1.2 murine metastatic breast cancer model *in vivo* to identify the most optimal synergistic combination of IVM for further clinical development

- Methods of enhancing integrated cancer immunotherapy will be investigated further to identify potential tumor eradication techniques coupled with immune-modulatory agents or chemotherapeutics with synergistic effects will be explored.

- Engineered OVA-expressing cells onto B16 melanoma or E0771 GFP/luciferase transfected breast cancer lines to investigate tumor specific T cell responses with OT-I/OT-II transgenic T cells. This will aid in future *in vivo* models to help evaluate tumor specific responses.

- DC vaccination potential will be investigated by combining therapeutic options identified by combined results of all tasks will be moved to *in vivo* analysis once rational approaches are determined *in vitro*.

Supporting Data/Figures:

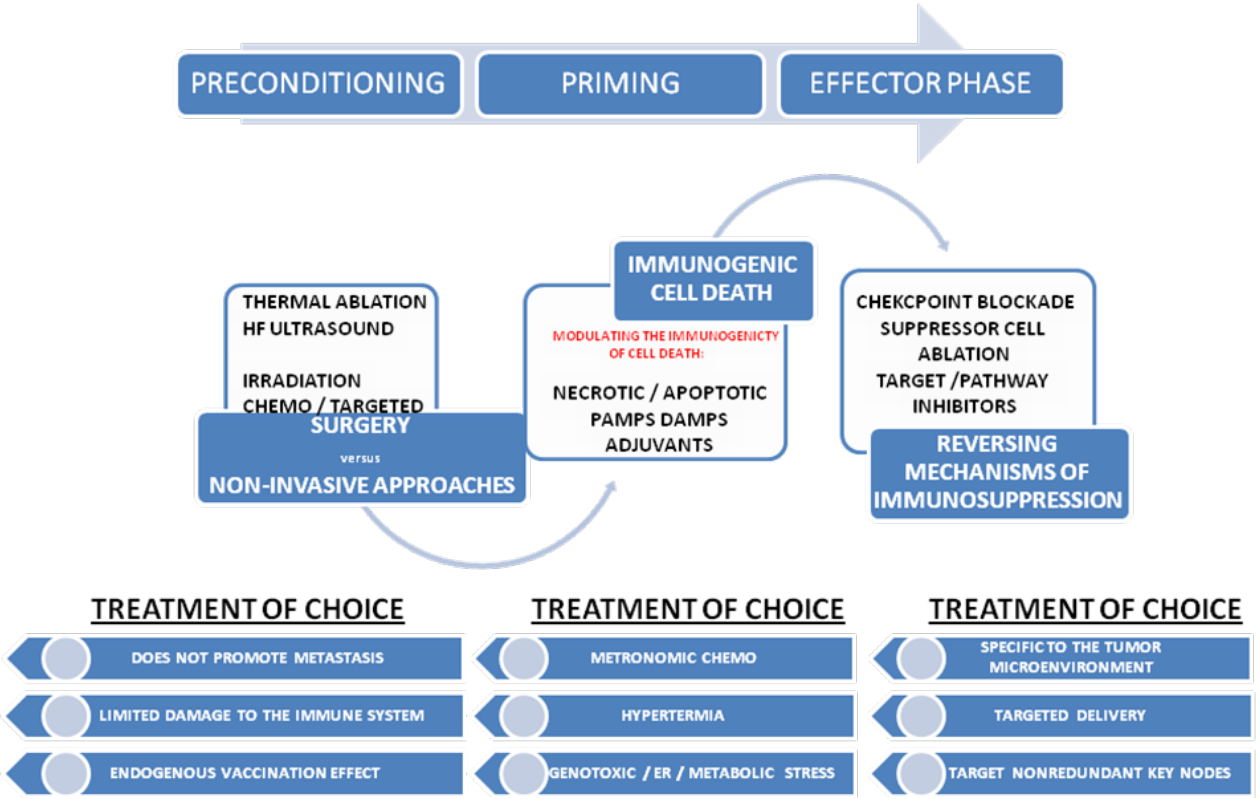


Figure 7: Platform for integrated cancer immunotherapy. Flow chart details the three main prerequisites for proper immune response and the potential approaches to investigate as we move forward with our *in vitro* and *in vivo* assays to develop integrated and combinatorial immunotherapy for cancer.

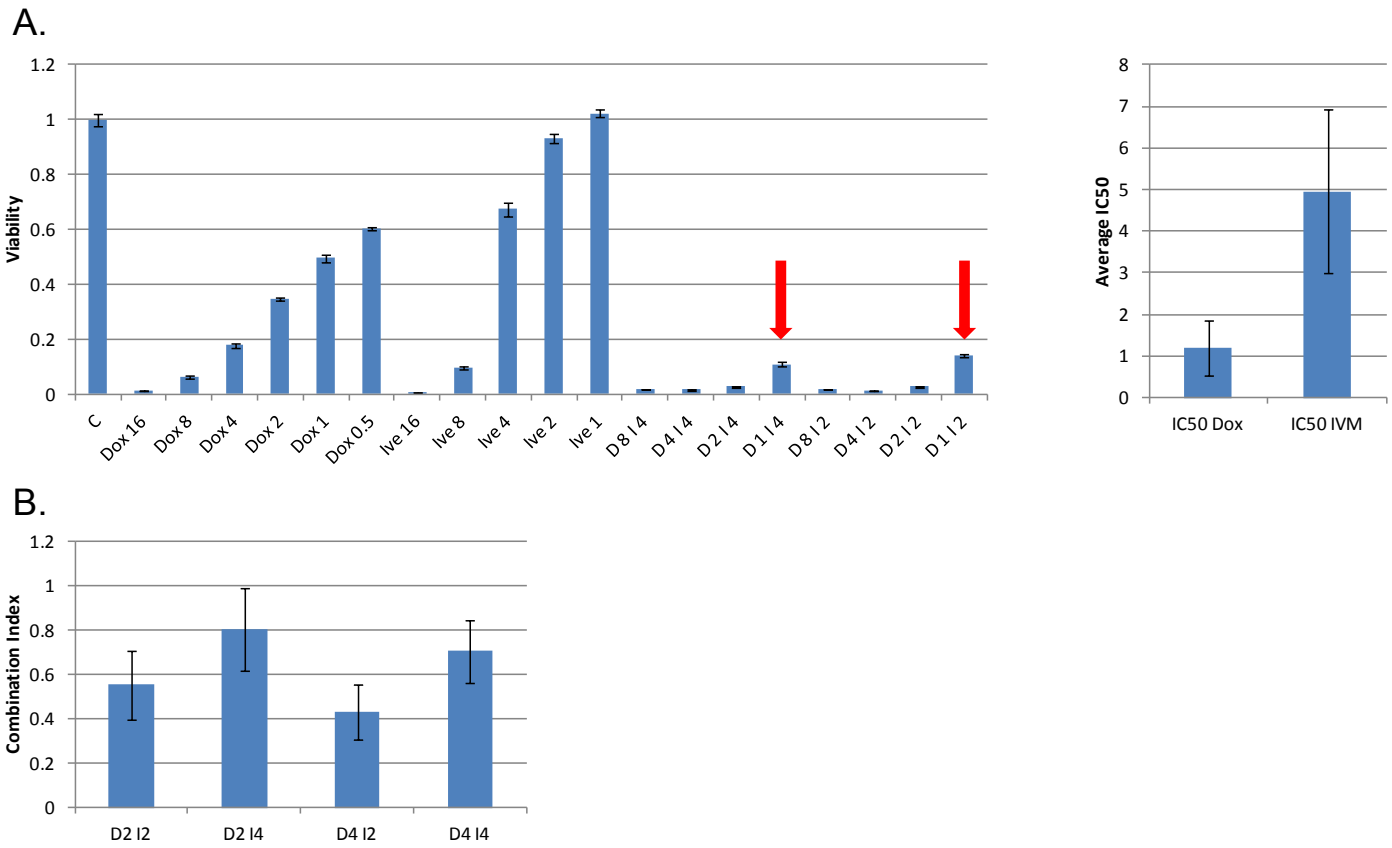


Figure 8. IVM and Doxorubicin synergistically kill triple negative 4T1.2 murine breast cancer cells. 4T1.2 cells were cultured for 48h with different doses of IVM and doxorubicin as indicated, and A) cell viability and IC50 were measured by the acid phosphatase (ACP) assay. B) Synergy quantification combination index values calculated using the Chou-Talalay method. CI is used to depict synergism (CI<1), additive effect (CI=1), and antagonism (CI>1). Data is representative of five independent experiments, in triplicate.

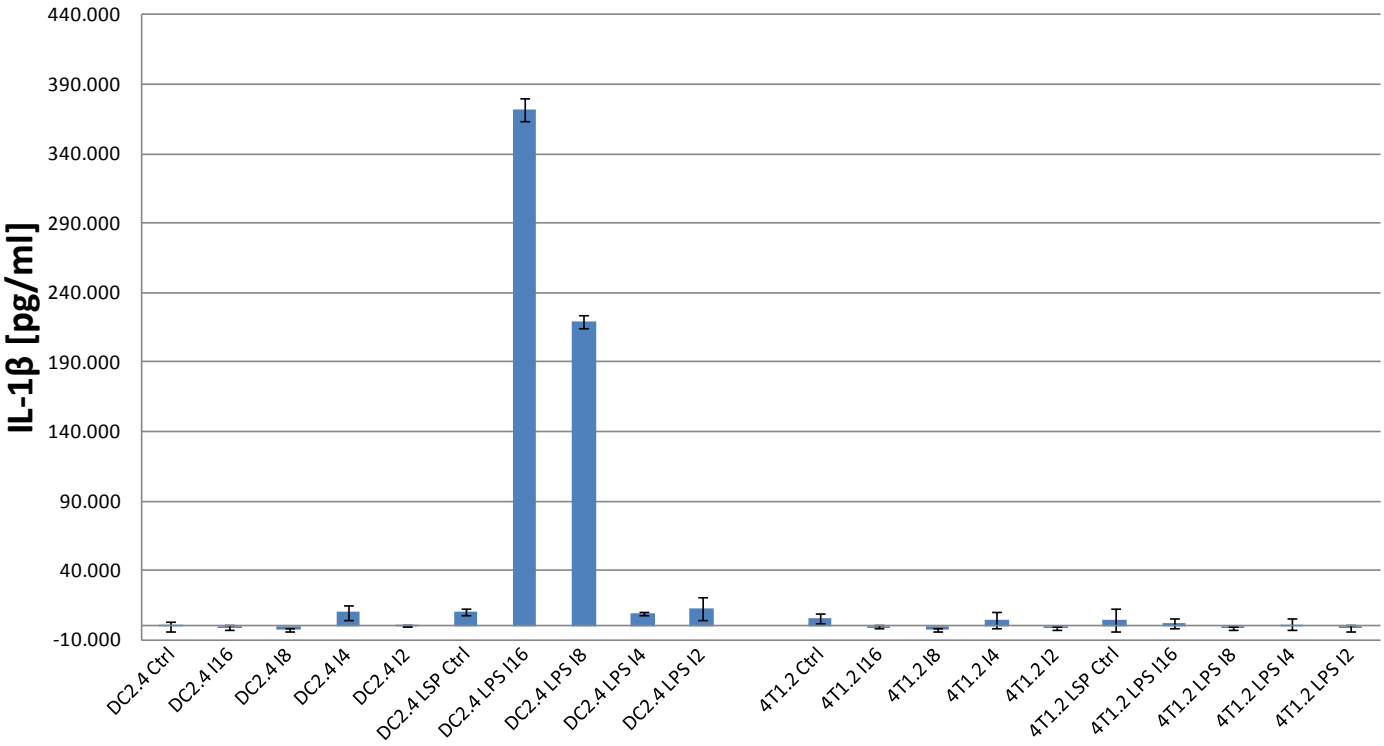


Figure 9. The ability of Ivermectin to potentiate purinergic signaling in antigen presenting cells and activate the inflammasome/caspase-1 pathway.

Murine DC2.4 (dendritic cell-like) and 4T.12 (breast cancer) cell lines were primed with LPS to induce IL-1 expression for 24h and were exposed to various doses of IVM to test for inflammasome activation and IL-1β processing and secretion. Supernatants were collected after 24h exposure and IL-1β secretion was measured by ELISA.

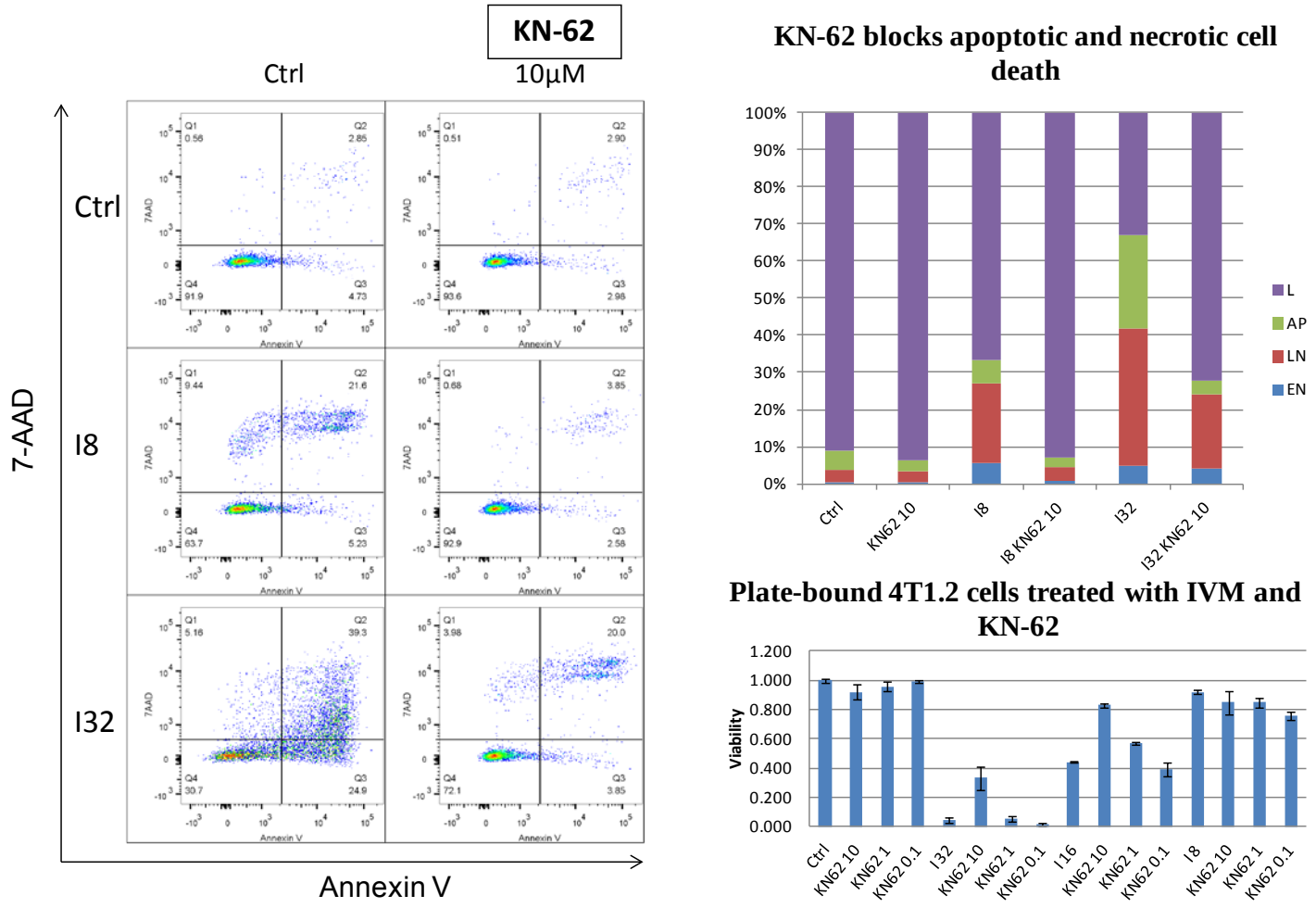


Figure 10. The P2X7 inhibitor KN-62 blocks IVM killing of 4T1.2 cells.
Plate-bound or suspension 4T1.2 tumor cells were exposed to various doses of IVM (µM) and the KN-62 inhibitor (µM) for 4h as indicated. Cell viability was evaluated by flow cytometry or the ACP assay.
(L: Live (Annexin V- 7-AAD-); AP: Apoptotic (Annexin V+ 7-AAD-); LN: Late necrotic (Annexin V- 7-AAD+); EN: Early necrotic (Annexin V+ 7-AAD+)).

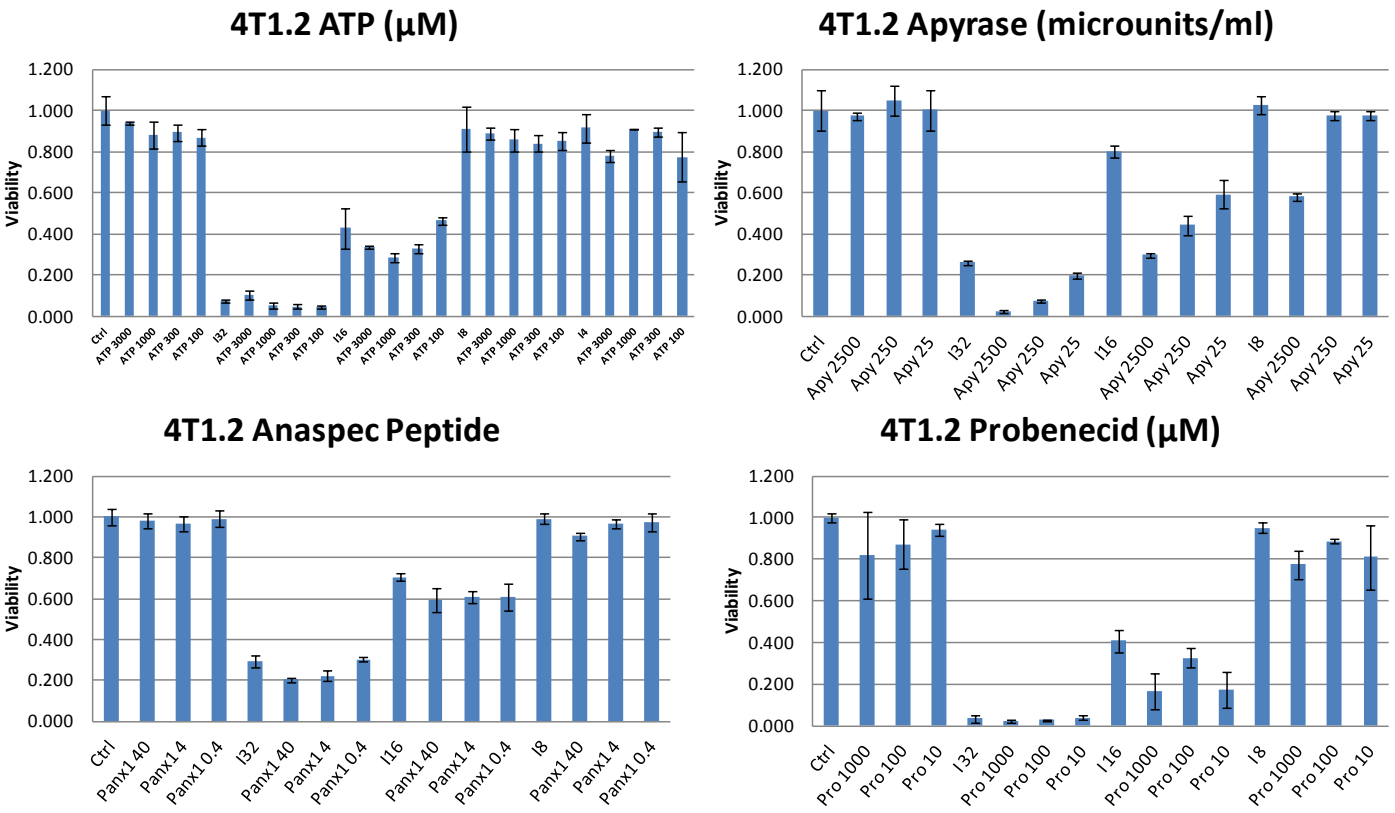


Figure 11. ATP plays a dual role in IVM-induced tumor cell death.
Plate-bound 4T1.2 cells were treated with increasing concentrations of IVM (µM) in the presence of exogenously added ATP, ATP degrading enzyme apyrase, or the pannexin-1 ATP release channel inhibitors, Anaspec peptide and Probenecid, as indicated. Cell viability after 4h incubation was evaluated by the Acid Phosphatase assay (ACP assay).

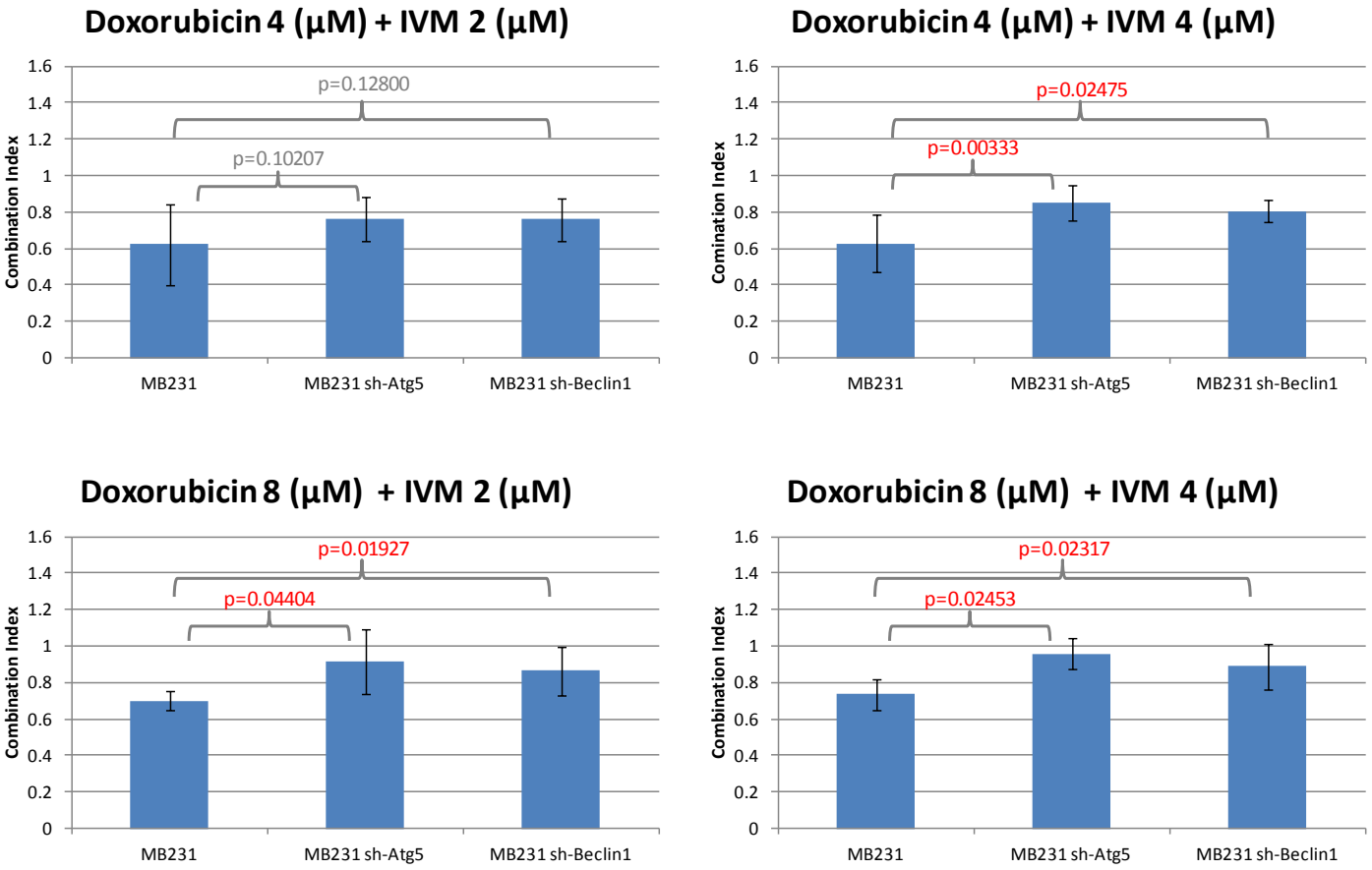


Figure 12. Synergy between doxorubicin and IVM is dependent on autophagy.
Wild type, Atg5, and Beclin-1 shRNA knockdown human triple negative breast cancer MDA-MB-231 cells were treated with IVM in combination with doxorubicin for 48h. Cell viability was evaluated by the ACP assay and the synergy between the drugs was compared after calculation of combinatorial indices. Data is representative of five independent experiments, in triplicate. P-values were determined by the paired T-test. P-values < 0.05 were considered significant.

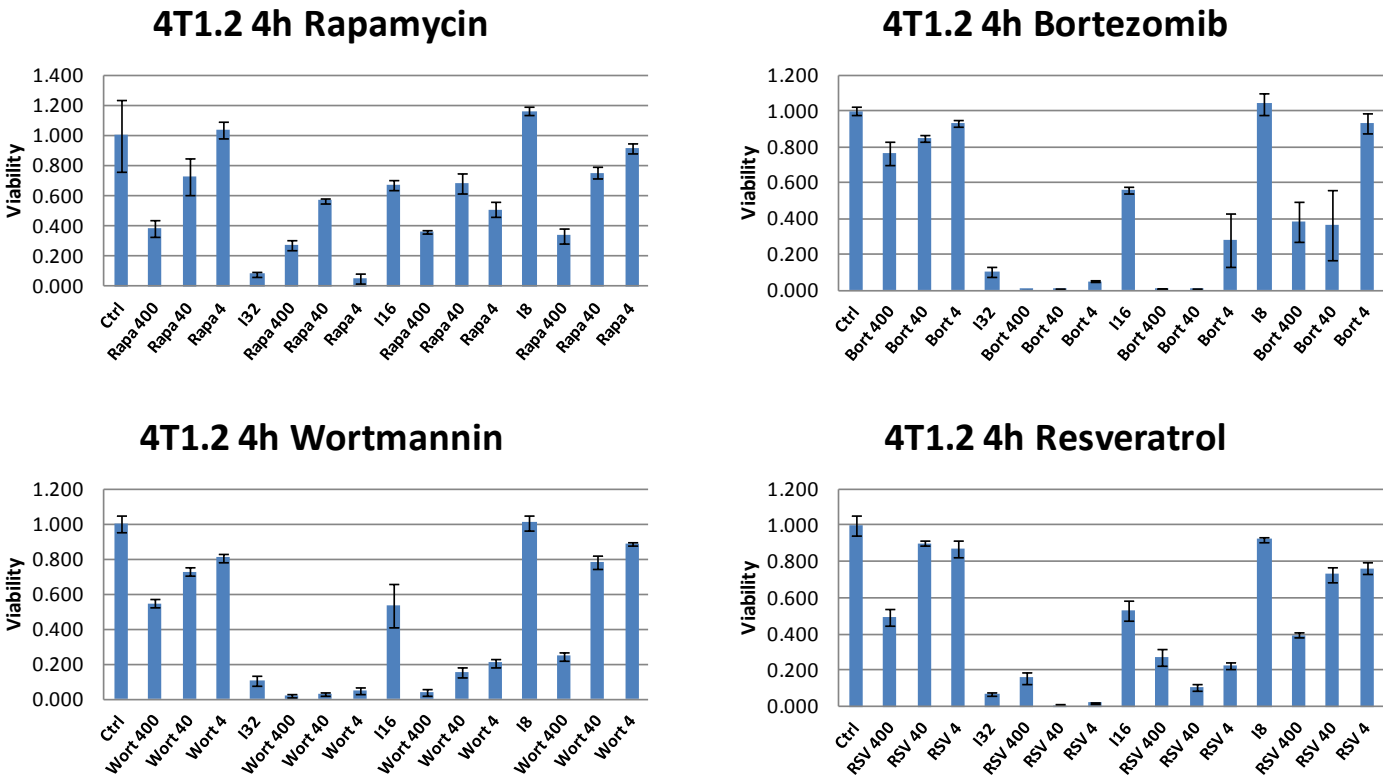


Figure 13. IVM synergizes with known inducers of autophagy.
4T1.2 cells were treated for 4h with IVM and Rapamycin, Bortezomib, Wortmannin, or Resveratrol. Cell viability was evaluated by the ACP assay.

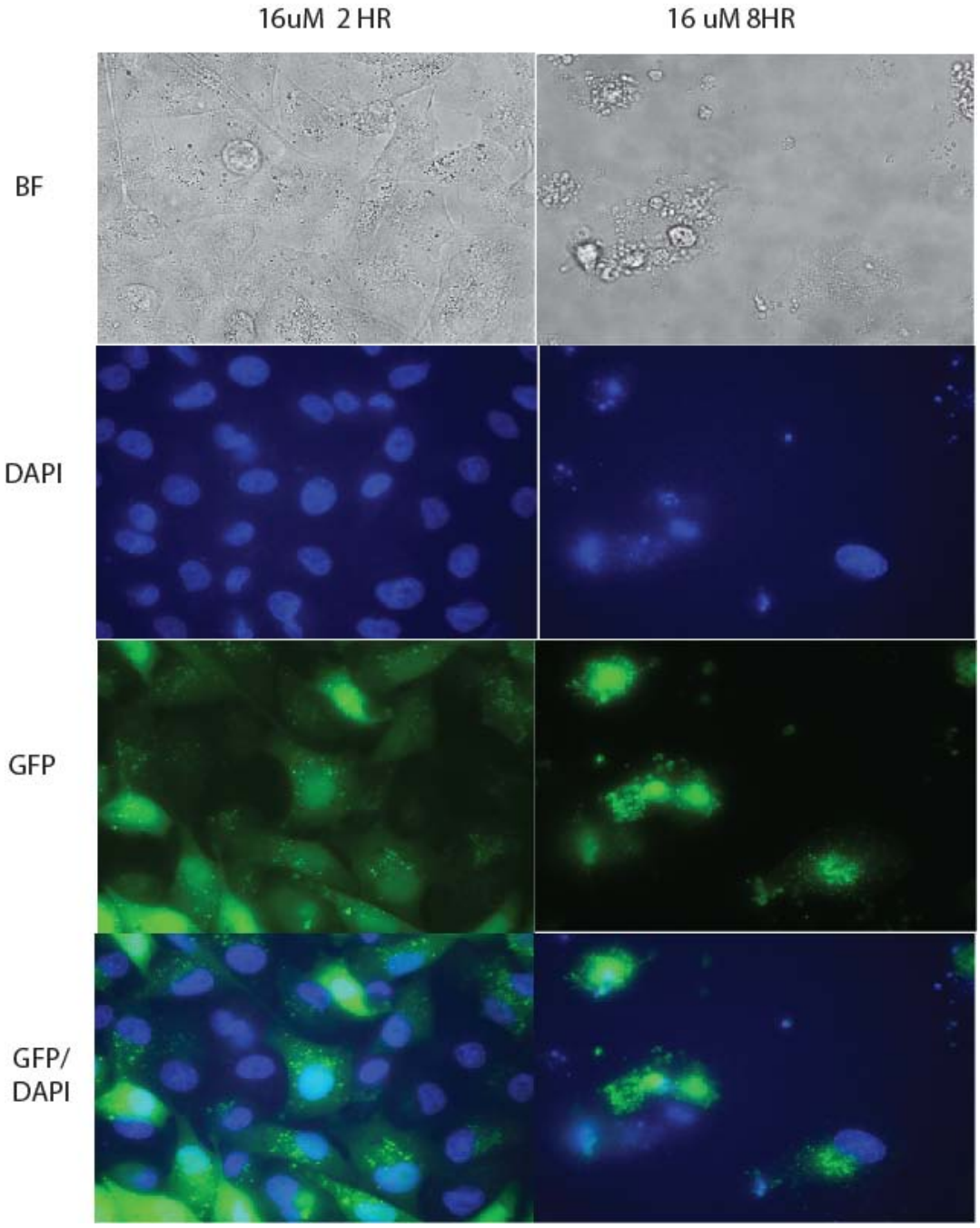


Figure 14. Induction of autophagy by IVM.
To detect autophagy, we used GFP-LC3 transfected MDA-MB-231 cells. Induction of autophagy in these cells caused the formation of visible high intensity GFP puncti. GFP-LC3 breast cancer cells were exposed to 8, 16, and 32 uM IVM for 1h, 2h, 4h, and 8h. Formation of GFP-LC3 puncti occurred in a dose and time dependent fashion. Shown is a representative bright-field (BF), DAPI (blue), GFP (green), and overlay staining of 16 uM IVM treated cells at 2h and 8h.

Hallmarks	Expected IVM Effects	Validation
ATP release	Likely Induces (necrotic cell death/swelling)	Pending formal validation
HMGB1 Release	Likely induces (necrotic cell death/swelling)	To test
Calreticulin Exposure	Enhanced	Up-regulated but marginal
M6P Exposure	Likely induces (autophagy dependent)	Up-regulated surface expression confirmed
H-60/Rae-1	Stress induced NKG2D ligands	Down-regulated

Figure 15. Summary of the hallmarks of immunogenic cell death (immunogenic apoptosis).
Preliminary findings regarding the effects of Ivermectin on specific immunogenic cell death characteristics is described in the validation column.

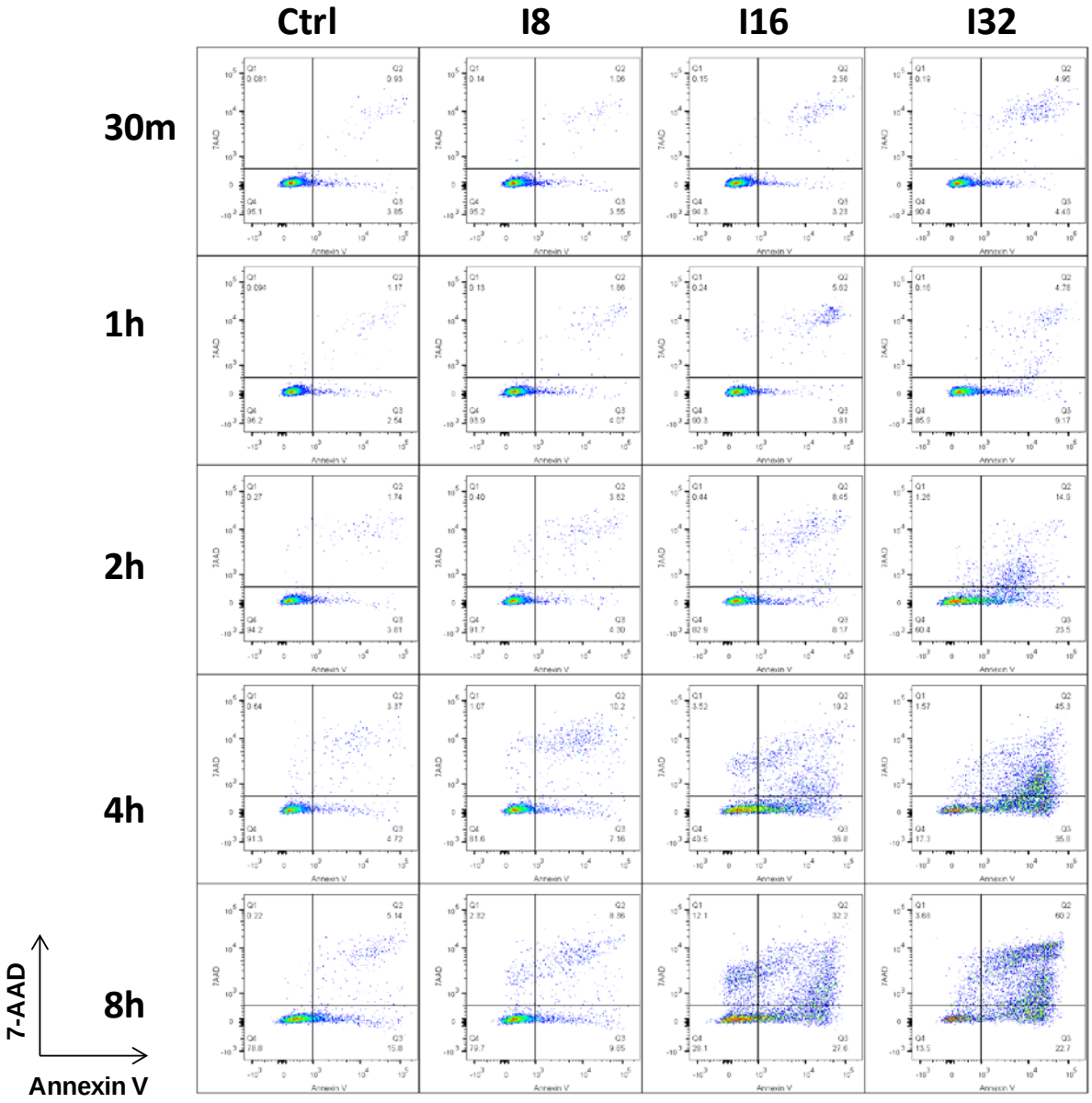


Figure 16. Time course of IVM-induced killing of 4T1.2 cells.
Suspended 4T1.2 tumor cells were treated with increasing doses of IVM and viability was analyzed by flow cytometry after staining with Annexin V and 7-AAD to discriminate between apoptotic and necrotic cells.

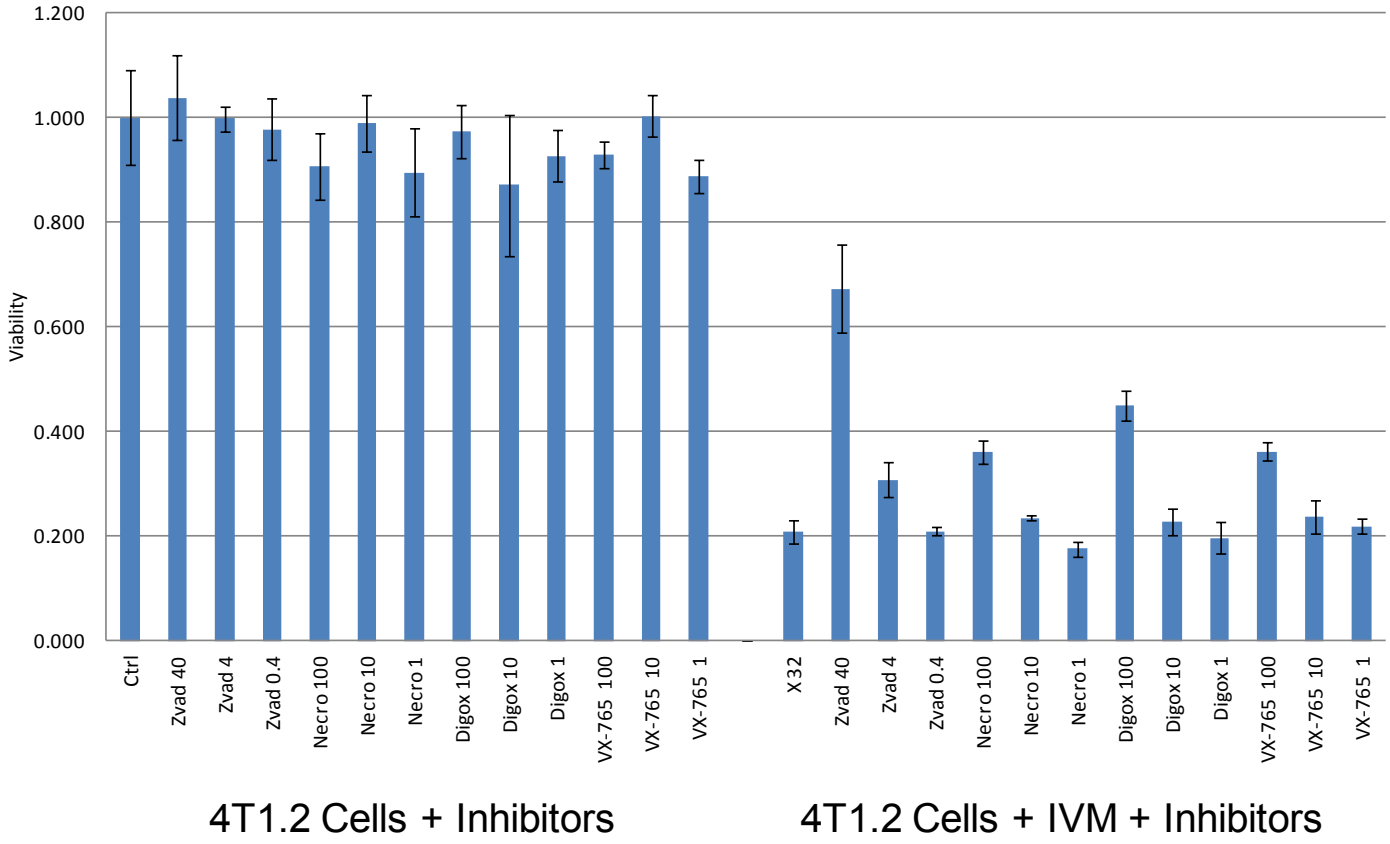


Figure 17. Involvement of various controlled cell death pathways in killing of 4T1.2 cells by Ivermectin. Plate-bound 4T1.2 cells were treated for 4h with high dose IVM to induce rapid cell death in the presence of inhibitors of apoptosis (Z-Vad-fmk), necrosis (necrostatin), autosis (digoxin), and pyroptosis (caspase-1). Cell viability was evaluated by the ACP assay.

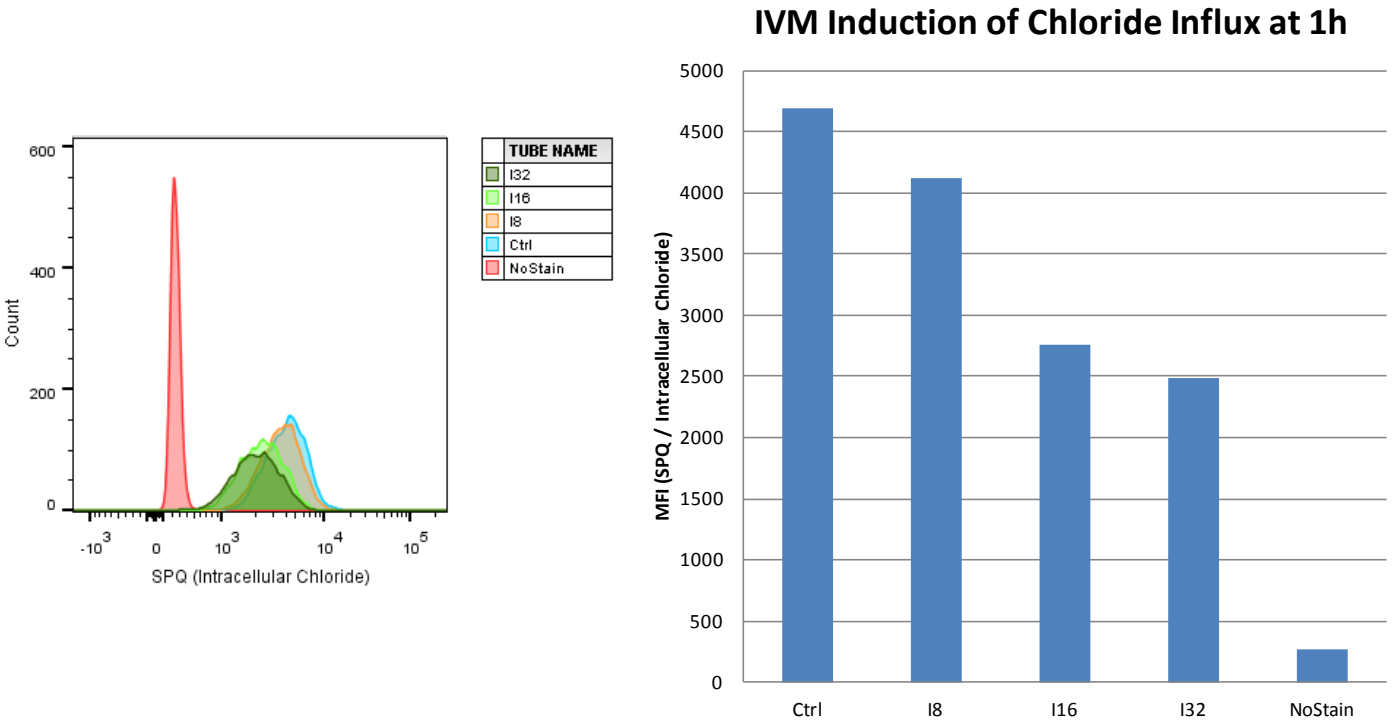


Figure 18. IVM induces Chloride influx into the cytosol, consistent with the cell size increase.
4T1.2 cells were loaded with the SPQ cytosolic chloride detection probe (and were exposed to increasing concentrations of IVM for 1h. Chloride influx (quenching of SPQ fluorescence) was detected by flow cytometry. (MFI: median fluorescent intensity).

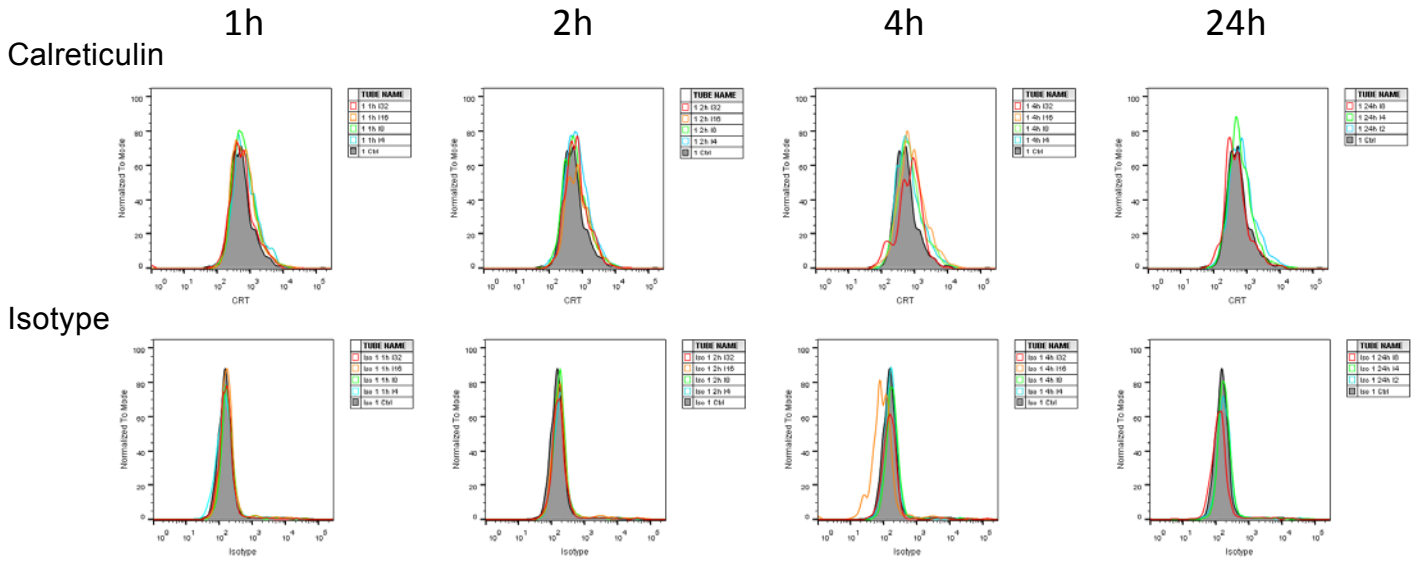


Figure 19. IVM up-regulates surface exposure of caltericulin (CRT).
MDA-MB-231 cells were treated with different doses of IVM and detached by enzyme free 1mM EDTA. CRT exposure was analyzed by flow cytometry after staining with CRT-specific antibody (top) versus Isotype control (bottom).

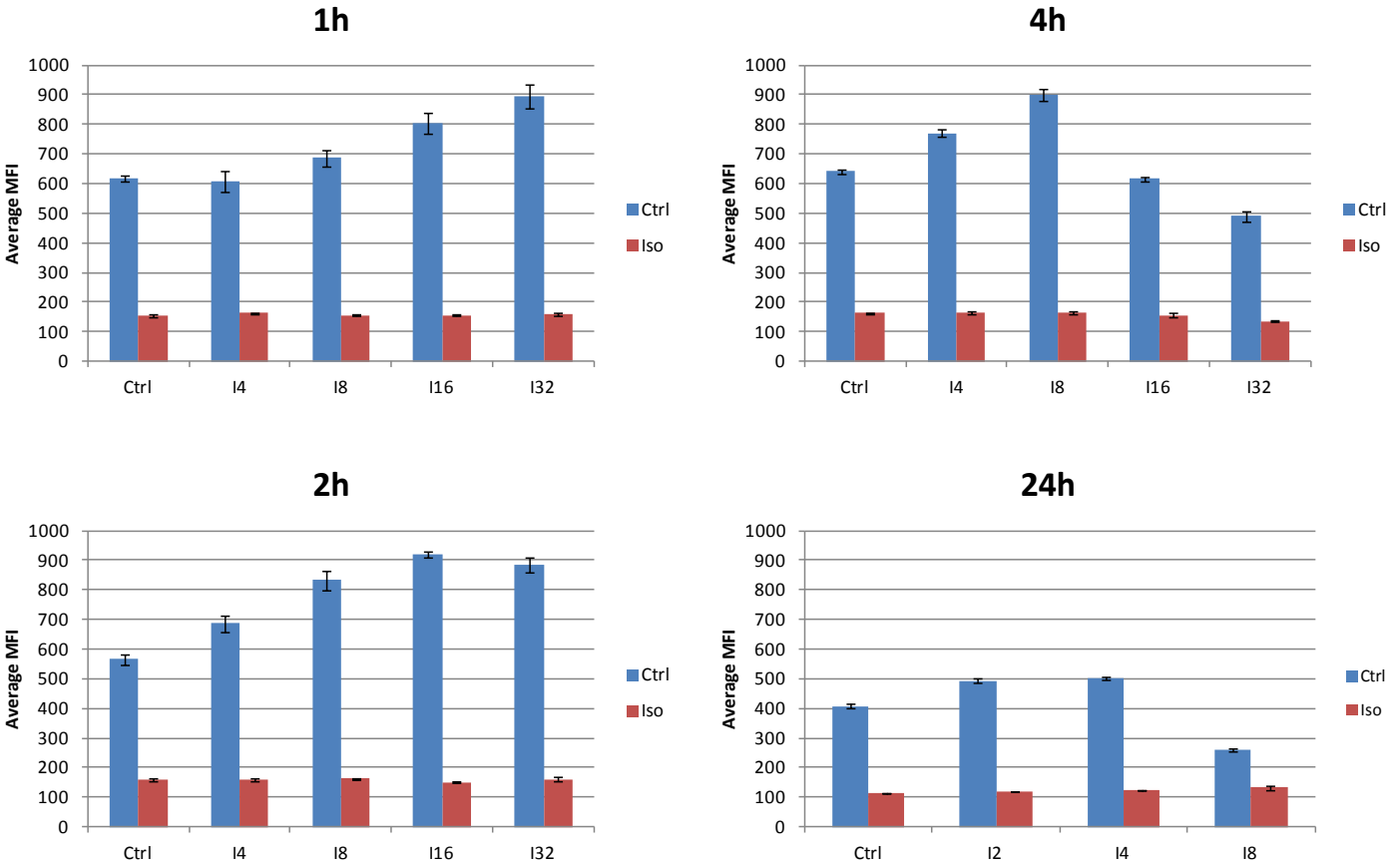


Figure 20. IVM up-regulates surface exposure of Mannose-6-phosphate (M6P).
MDA-MB-231 cells were treated with different doses of IVM and detached by enzyme free 1mM EDTA. M6P exposure was analyzed by flow cytometry after staining with M6P-specific antibody (blue) versus Isotype control (red). (MFI: Median fluorescent intensity)

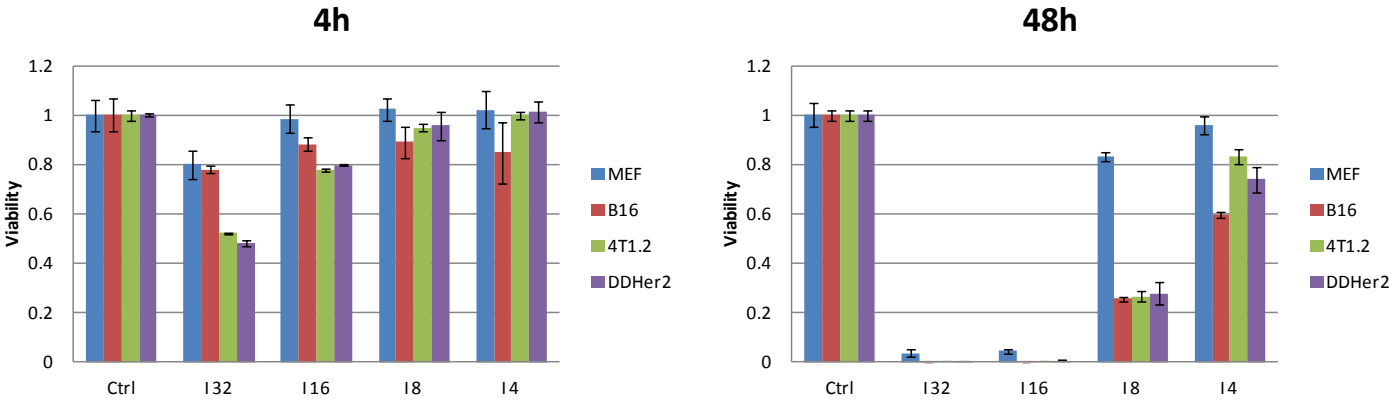


Figure 21. Tumor specificity of IVM.
Murine mouse embryonal fibroblasts (MEF), B16 melanoma cells, as well as 4T1.2 (triple negative) and DDHer2 (Her-2 positive) breast cancer cells were treated with increasing concentrations of IVM for 4h and 48h. Cell viability was evaluated by the ACP assay as above.

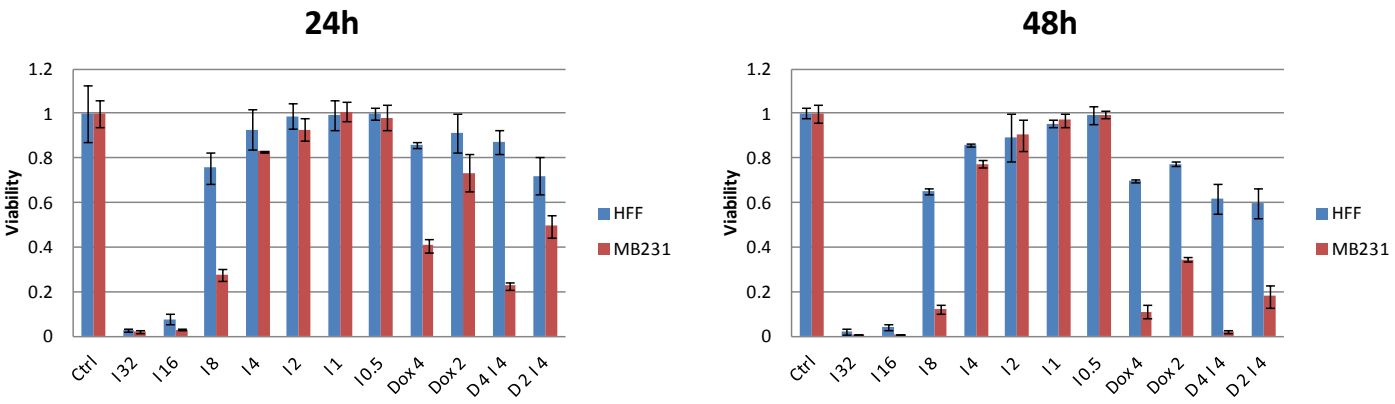
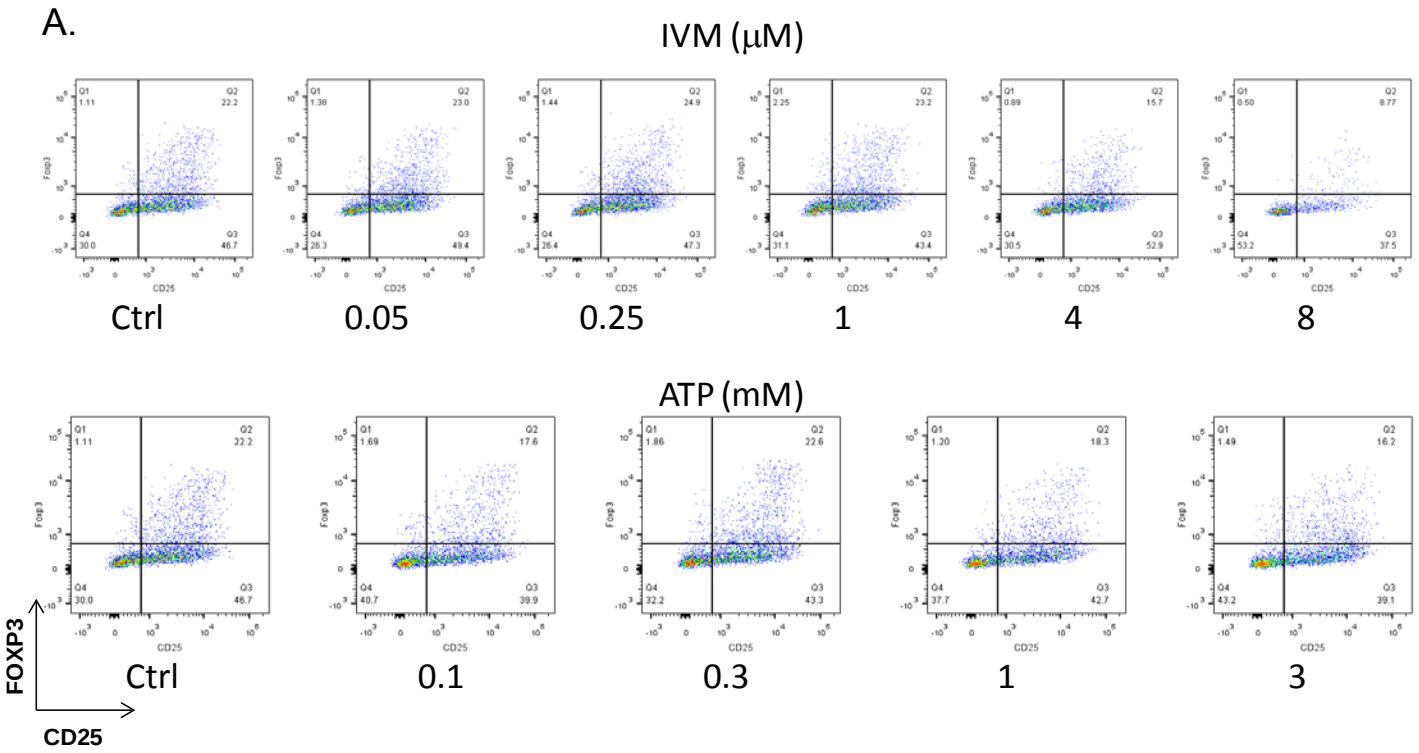


Figure 22. Tumor specificity of the Doxorubicin and IVM combinations.
Human foreskin fibroblasts (HFF) and MDA-MB-231 triple negative breast cancer cells were treated with increasing concentrations of IVM and/or doxorubicin for 24h and 48h. Cell viability was evaluated by the ACP.



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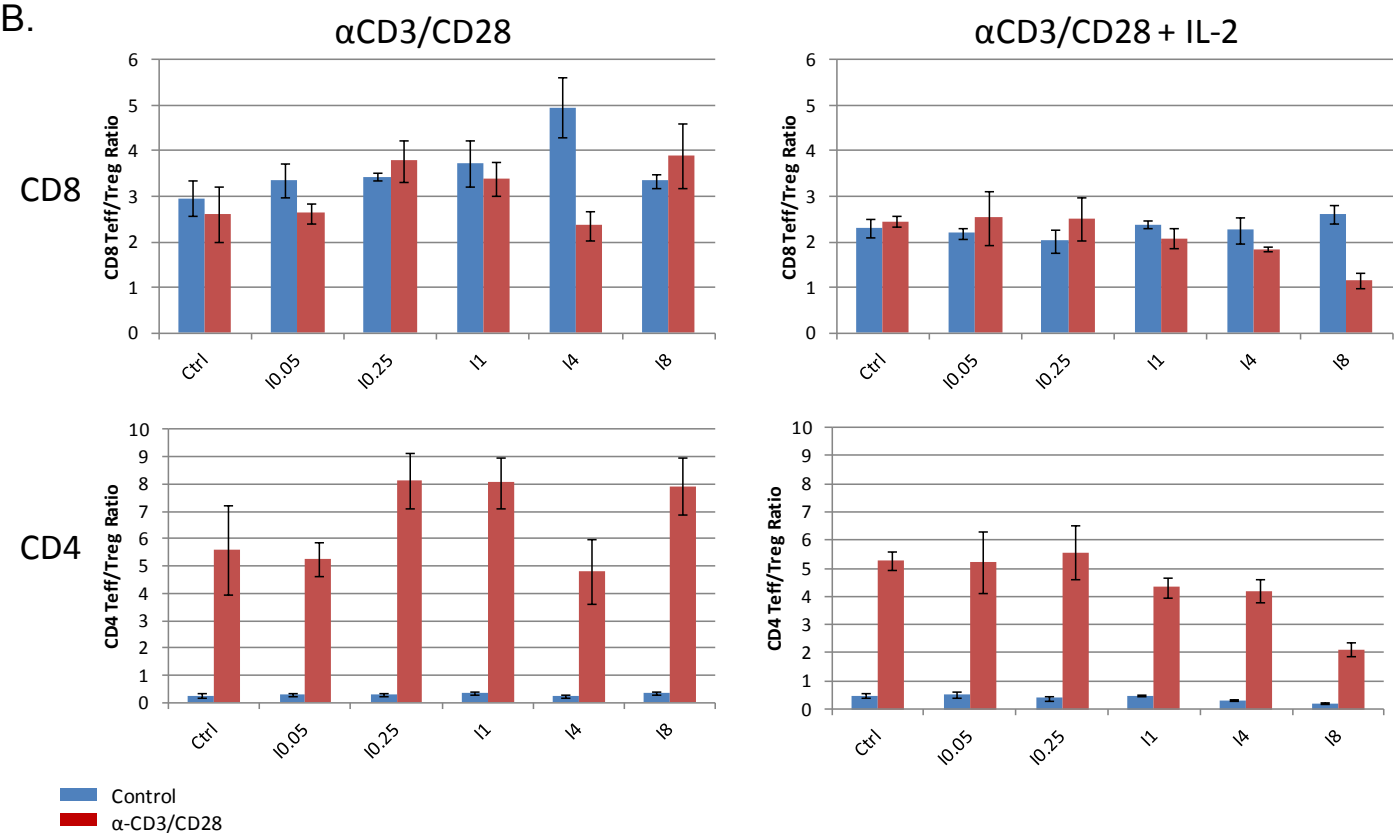
αCD3/CD28
αCD3/CD28 + IL-2
CD8
CD4
CD8 Teff/Treg Ratio
CD4 Teff/Treg Ratio
Ctrl
I0.05
I0.25
I1
I4
I8
Control
α-CD3/CD28

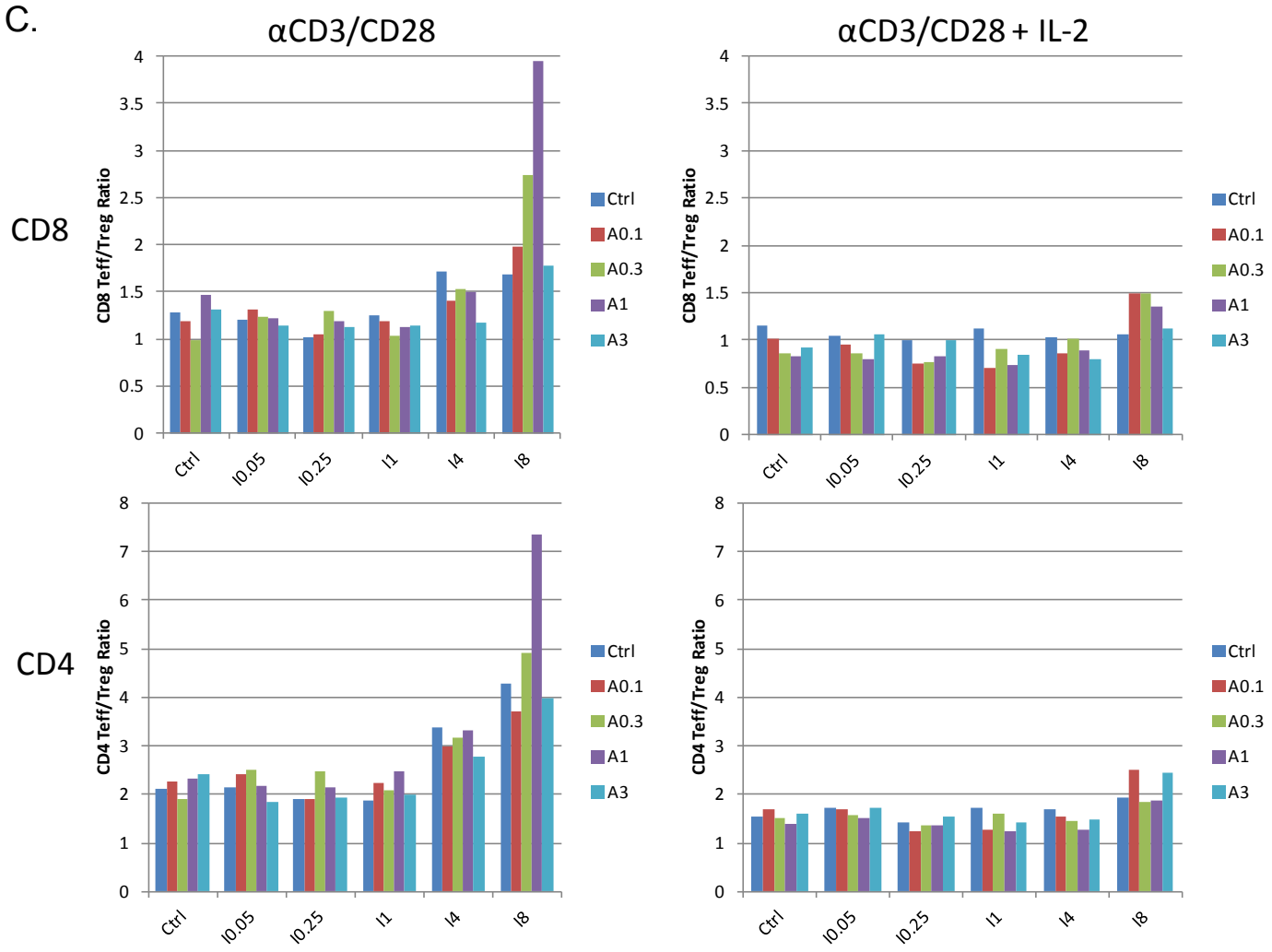

Figure 23. IVM rather than ATP enhanced the Teff/Treg ratio in ex *vivo* stimulated CD4+ T cells.
PBMCs treated with increasing concentrations of ATP and IVM. PBMCs were stimulated with soluble αCD3/CD28 antibodies for 4h. Flow cytometry was used to analyze the fraction of Teff and Tregs after staining for CD4, CD25 (surface) and Foxp3 (intracellular). (A) representative flow cytometry analysis. (B) Quantification of the effect of IVM on CD8+ Teff/Treg and CD4+ Teff/Treg ratios in the presence or absence of IL-2. (C) Quantification of the combination of IVM and ATP on CD8+ Teff/Treg and CD4+ Teff/Treg ratios in the presence or absence of IL-2.

KEY RESEARCH ACCOMPLISHMENTS:

- Substantial progress has been made in successfully growing breast cancer patient-specific cancer cells and CAS (from both primary and metastatic tumors), and using these cells to generate 3D organoids

- Integration of multiple cell types involved in *in vitro* 3D tumor microenvironment, especially tumor associated stromal cells, to determine factors which affect tumor progression and metastasis.

- Continued optimization of a novel 3D cell culture approach to expand and re-aggregate subpopulations of primary human breast cancer tumor, stromal and immune cells, which are critical for supporting or inhibiting DC clustering and maturation as well as cancer growth.

- Banked RNA of various stromal cell types from both healthy and tumor associated tissues to determine molecules or genetic expression profiles for future analysis.

- Identified Ivermectin (IVM) as a potent agent that can kill mouse and human breast cancer cells alone and is also highly synergistic in combination with Doxorubicin.

- Demonstrated that IVM to modulate purinergic signaling and IVM killing could be effectively blocked by the specific P2X7 inhibitor KN-62

- Investigated and demonstrated that autophagy and ATP release are directly involved in the synergy between Doxorubicin and Ivermectin and that several known inducers of autophagy were able to synergize with IVM in killing of 4T1.2 cells.

- Established the therapeutic window and the tumor specificity of IVM treatment.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

None at this time – studies ongoing.

CONCLUSION:

We have a strong research team for this project, which includes assistant research professor Dr. Brile Chung, PhD postdoctoral fellows Dr. Dobrin Draganov and Dr. Neta Zuckerman, and research associates Sara Moeller and Gayathri Srinivasan. We have worked closely with the CoH IACUC office on animal protocols which one of two has been approved. We continue to develop an in-depth understanding of the immune system within the setting of the tumor microenvironment. We have made progress in developing methods to better analyze the relationships between primary tumor and metastatic growths with their surrounding microenvironment and implications for immune response and dendritic cell function *in vitro* using 3D microculture techniques. We have further investigated and tested alternative methods of combinatorial drugs in attempts to eradicate cancer cells while preserving the immune system. We are well positioned to gain further insights in the next 12 months that will aid in our goal of restoring long term immune function in breast cancer patients to optimal levels, and subsequently eradicate metastases to prevent relapse in breast cancer patients.

APPENDICES:

None at this time.

PERSONNEL:

Peter P. Lee, MD – PI	25% Effort
Brile Chung, PhD – Assistant Research Professor	100% Effort
Dobrin Draganov, PhD – Post Doctoral Fellow	100% Effort
Neta Zuckerman, PhD – Post Doctoral Fellow	50% Effort to 3/22/14
Emily Andersen - Research Associate I	50% Effort to 6/13/14
John Murad - Research Associate I	100% Effort to 7/18/14
Sara Moeller, Research Associate I	50% Effort since 6/23/14
Gayathri Srinivasan, Research Associate I	100% Effort since 7/28/14

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